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THE BIOLOGY OF THE AMOEBA

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* This series of papers is the result of a conference on *The Biology of the Amoeba* held by The New York Academy of Sciences, March 24 and 25, 1958.

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ROBERT CHAMBERS: MICRURGIST AND CELL PHYSIOLOGIST

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Robert Chambers, research professor of biology at New York University from 1928 to 1947 and professor emeritus of biology from 1947, died at Concord, N. H. on July 22, 1957.¹⁻³ Chambers was born in Turkey on October 23, 1881, the son of Canadian Presbyterian missionaries. Most of his youth was spent in the Middle East where, as a student in elementary schools, his interest in natural history was fostered. In 1900, he earned his Bachelor of Arts degree at Roberts College in Istanbul. He received a Master of Arts degree from Queen's College in Kingston, Canada in 1902 and, in 1944, he was awarded the honorary Doctor of Letters degree. Chambers migrated from Canada to Europe in search of further graduate training. He earned his doctorate in 1908, as a student of Richard Hertwig at the University of Munich, Munich, Germany. Chambers was a favorite student of Hertwig and unquestionably the seeds of interest in living cells were firmly planted through Hertwig's teachings and inspiration.

A most fortuitous association occurred at the Marine Biological Laboratory in Woods Hole, Mass., during the summer of 1912. There Chambers met George L. Kite, who was applying new techniques to the study of living cells with the hope that the physical properties of the living stuff known as protoplasm could be determined. Kite was using the Barber Pipette Holder, a micromanipulator, in his studies on cells and thus the application of microdissection techniques to cells was seriously launched. Chambers loved adventure, and this new microdissection technique was the challenge he needed. That summer, Kite and Chambers⁴ collaborated on a study of the function and structure of the nucleus. They were able for the first time to dissect chromosomes out of cells.

Although Kite's productive years were abruptly shortened by ill health, Chambers continued the research in microdissection of cells and became the unquestioned master of micrurgy. A decade later he developed new instruments and methods that made possible, not only exquisitely precise dissection of living cells, but other operations as well, even under the highest powers of the microscope. A major contribution of Chambers was the simplification of procedures and instrumentation for microinjection of fluids into living cells. To these instruments he added the motion picture camera, so that many interesting and significant experiments were recorded for all time and for everyone to see. Even today, some of these cinematographic records are classic examples of excellent micrurgy and motion picture technique.

Chambers came to New York University in 1928 from Cornell University Medical College, where he was professor of microscopic anatomy. For almost 30 years, Chambers served his university and his adopted country with distinction. It was here that his manifold and pioneering studies on protoplasm, the students he trained, and the scientists, especially the younger ones, with whom

he collaborated, established his international reputation as one of the foremost authorities in the field of experimental cell research. Chambers' greatest fame came, not only as the result of developing the art and science of microsurgery, but through his imaginative applications of these techniques to the solution of some of the mysteries of living cells. For these contributions, Chambers received the Traill Medal of the Linnean Society of London, England, the John Scott Medal from the City of Philadelphia, Pa., and election as Honorary Fellow of the Royal Microscopical Society, London.

Much of Chambers' work and of his students and associates was on the amoeba. In Chambers' laboratory at New York University, for many years the amoeba was the material of choice for study—and it was replaced by echinoderm eggs only during the summers at Woods Hole. The pioneering studies on the effects of salts and other substances on the amoeba⁵ began to yield the first experimental information on the colloidal properties of protoplasm. These studies demonstrated the differential effects of applying certain substances to the surface of cells (by immersion) or to the interior (by microinjection).

The influence of Chambers on this publication is unquestionable; many of the papers have been presented by his former students. Other contributors have been "second generation" students. Chambers would have thoroughly enjoyed reading this monograph and would have been one of its most distinguished contributors. It is appropriate, then, that this publication on the biology of the amoeba be dedicated to his memory.

References

1. DURYEE, W. R. 1957. R. Chambers: pioneer in the study of living cells. *Science*. **126**: 645.
2. ZWEIFACH, B. W. & G. H. A. CLOWES. 1958. Memorial: Robert Chambers. *Biol. Bull.* **115**: 10.
3. MARSLAND, D. A., M. J. KOPAC & H. A. CHARIPPER. 1958. Robert Chambers, Hon. F. R. M. S. *J. Roy. Microscop. Soc.* **77**: 86.
4. KITE, G. L. & R. CHAMBERS, JR. 1912. Vital staining of chromosomes and the function and structure of the nucleus. *Science*. **36**: 639.
5. CHAMBERS, R. & P. REZNIKOFF. 1926. Micrurgical studies in cell physiology. I. The action of the chlorides of Na, K, Ca, and Mg on the protoplasm of *Amoeba proteus*. *J. Gen. Physiol.* **8**: 369.

INTRODUCTION

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In 1950 Robert Chambers acted as consulting editor of a monograph entitled *Structure in Relation to Cellular Function*¹ after having served as chairman of the conference on which the publication was based. With the single exception of the contribution made on this occasion by Heinz Holter,² the large, free-living amoebae received only casual mention. Although studies of *Amoeba* reported by R. Chambers, H. W. Chalkley, S. O. Mast, and others were receiving increasing recognition, *Amoeba proteus* was primarily known as pedagogic material for work in courses in elementary biology.

During the past decade there has been a growing awareness of the potential value of these forms in investigations into the nature of the cell, for their size and nuclear-cytoplasmic arrangements have made them almost ideally suited for such studies. In this short period of time, the large, free-living amoebae have provided substantial basic information, notably in the areas of cytochemistry and nuclear function. Studies of amoeboid movement have led to important advances in the understanding of cytokinesis through reversible sol-gel transformations. Various enzymes have been localized in the *Amoeba*. The experiments on nuclear-cytoplasmic relationships and nuclear transplantation emphasize the future importance of these forms in the field of cytogenetics. The systematics of the large, free-living amoebae has been improved by the studies of R. R. Kudo, and the nomenclature has been clarified.

These developments only indicate the growing interest in the two protozoan genera that have become increasingly important in biological research, *Amoeba* and *Pelomyxa* (Chaos). To refer once more to the earlier 1950 monograph, today it would be relatively easy to devote a conference and a monograph to these two genera alone.

However, the primary purpose of the conference on which this monograph is based was not to investigate the field of structure and function, important as these aspects may be, but rather to explore from a variety of viewpoints the wider current knowledge of these organisms. Due to the different areas of interest of the investigators utilizing these forms, their contributions generally appear in widely scattered journals. It was felt that a conference could provide a suitable unified framework for expression by active investigators in representative areas of research.

One of the successfully accomplished objectives of the conference was that it assembled interested investigators from different areas for the purpose of furthering acquaintance and communication. Another purpose, fulfilled, it is hoped by this publication, is to obtain for a larger audience a functional inclusive reference source for these forms. In addition, this monograph, representing the results of combined efforts, will serve its purpose if it acquaints those who are familiar with the amoebae only as classroom material with the potential value of these organisms in their own research.

Since many of the contributions to this publication are from former students of Robert Chambers or from their students, and since so much of the rest of its contents has its origin in his work, it seems particularly fitting that this monograph should be dedicated to him.

References

1. CHAMBERS, R. 1950. Ann. N. Y. Acad. Sci. **50**(8).
2. HOLTER, H. 1950. Ann. N. Y. Acad. Sci. **50**(8): 1000.

Part I. Structural and Taxonomic Considerations

THE CYTOLOGY OF *PELOMYXA**

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During recent years *Amoeba proteus* and *Pelomyxa carolinensis* have achieved important status as experimental animals in various areas of research. These amoebae offer several advantages as material for cytological study. Among these would be ease of culture and the comparatively large size of the organisms, which facilitates observation. Mast and Doyle (1935, 1935a) did the classic work on the cytology of *A. proteus*, while the general cytology of *P. carolinensis* has been treated in a series of papers (Andresen, 1942, 1956; Wilber, 1942, 1945; Kudo, 1946, 1947). The cytoplasmic inclusions found within the two organisms appear to be similar in all respects with the exception of the nuclei, *Pelomyxa* being multinucleate. Kudo (1947) has given a complete description of the nuclei and nuclear division in *P. carolinensis*.

This paper is a continuation of earlier work on the cytology of *Pelomyxa* (Torch, 1955) and is concerned with the effects of centrifugation on the organisms, the neutral red system, and the reaction of the organisms to various Golgi techniques.

Materials and Methods

Organisms were maintained in glass-covered finger bowls in an incubator at 22° C. The culture medium consisted of Pyrex distilled water to which two rice grains were added. Paramecia were added every other day, and the cultures were subcultured every two weeks.

Centrifugation. The organisms were centrifuged in 15-cc. centrifuge tubes at 700 × gravity for 45 min. in an International Refrigerated Centrifuge kept at 0°C. A 20 per cent gum arabic solution was used as the suspension medium. In calculating the relative centrifugal force (rcf) according to the formula $rcf = 0.0000118 \times \text{radius} \times \text{rpm}^2$, the radius employed was the distance from the center of the rotor to the top of the gum arabic solution. The organisms were layered over the gum arabic solution, 2 or 3 cc. of Pyrex distilled water was added, and then the tube was rolled back and forth vigorously between the palms of the hands. The latter procedure establishes a gradient of specific gravities within the centrifuge tube. Centrifuged organisms were placed in Syracuse watch glasses containing a thin layer of water over an ice base. The organisms could be kept immobile as long as ice persisted within the dish. In making permanent preparations, centrifuged organisms were placed into partially frozen chromic acid-osmium fixative (1 per cent chromic acid; 2 per cent osmium tetroxide, equal parts) or into ice cold 4 per cent formalin. Fixed organisms were embedded in Tissuemat (56 to 58° C.) and

* This investigation was supported in part by Research Grant C-2522 from the National Cancer Institute, Public Health Service, Bethesda, Md.

sectioned at 6 μ . The sections were deparaffinized and mounted either unstained or stained in Heidenhain's iron hematoxylin with picric acid differentiation. The technique for handling the organisms through the various solutions has been described (Torch, 1955).

Neutral red. Vital staining of the organisms was accomplished in Syracuse watch glasses containing 10 cc. of aqueous solutions of neutral red (1:100,000 w/v). Organisms were selected at varying intervals, rinsed in Pyrex water, and observed under the microscope. Permanent preparations were obtained by fixation in chromic acid-osmium, embedding in Tissuemat (56 to 58° C.), and sectioning at 6 μ .

Golgi techniques. The osmium techniques of Mann-Kopsch, Nassanov, and Hirschler (McClung, 1950) were employed, as well as the silver methods of Aoyama (Baker, 1945), Da Fano, and Cajal (McClung, 1950). Some material was osmicated for periods of up to 8 days at 35° C. after fixation in chromic acid-osmium. After embedding, sections were cut at 6 μ , and the sections were mounted without subsequent staining.

Observations

Centrifuge studies. After centrifuging at 700 g for 45 min., stratification within the organism can be readily detected with the naked eye. When placed in ice water, many of the animals become oriented perpendicular to the bottom of the container with the heavy ends directed downward. The centripetal end of each animal is generally much broader than the centrifugal end. Some organisms retain their pseudopodia, and stratification is evident within the individual pseudopods. Examination with the compound microscope reveals the following arrangement of cytoplasmic strata (FIGURE 1).

Packed into the centripetal end are fat droplets, contractile vacuoles (many containing a mitochondrial coat in a centrifugal position), and small vacuoles, which are interpreted as crystal vacuoles. The fat droplets give a brownish cast to the cytoplasm when viewed by transmitted light. Centrifugal to the small vacuoles is a large hyaline area within which are located a few crystals and small food vacuoles. The hyaline region accounts for approximately one half of the volume of the centrifuged organism. The middle of the hyaline layer generally occurs midway between the poles of the centrifuged organism.

Just centrifugal to the hyaline area is found the centrifugal end of the organism into which are packed the remaining inclusions. This region appears brownish-black with transmitted light. Proceeding centrifugally, the following layers can be identified: crystals within vacuoles, crystals not enclosed within vacuoles, nuclei, mitochondria, food vacuoles, and refractive bodies. The mitochondrial and food vacuole layers overlap, and generally there is an abundance of crystal vacuoles distributed throughout the centrifugal end. The alpha granules are not confined to a single stratum, but are concentrated in the centrifugal end. Centrifugation at slower speeds (340 g) yields identical results.

These observations are in essential agreement with those of Andresen (1942). Mast and Doyle (1935a) and Singh (1938) reported stratification within the

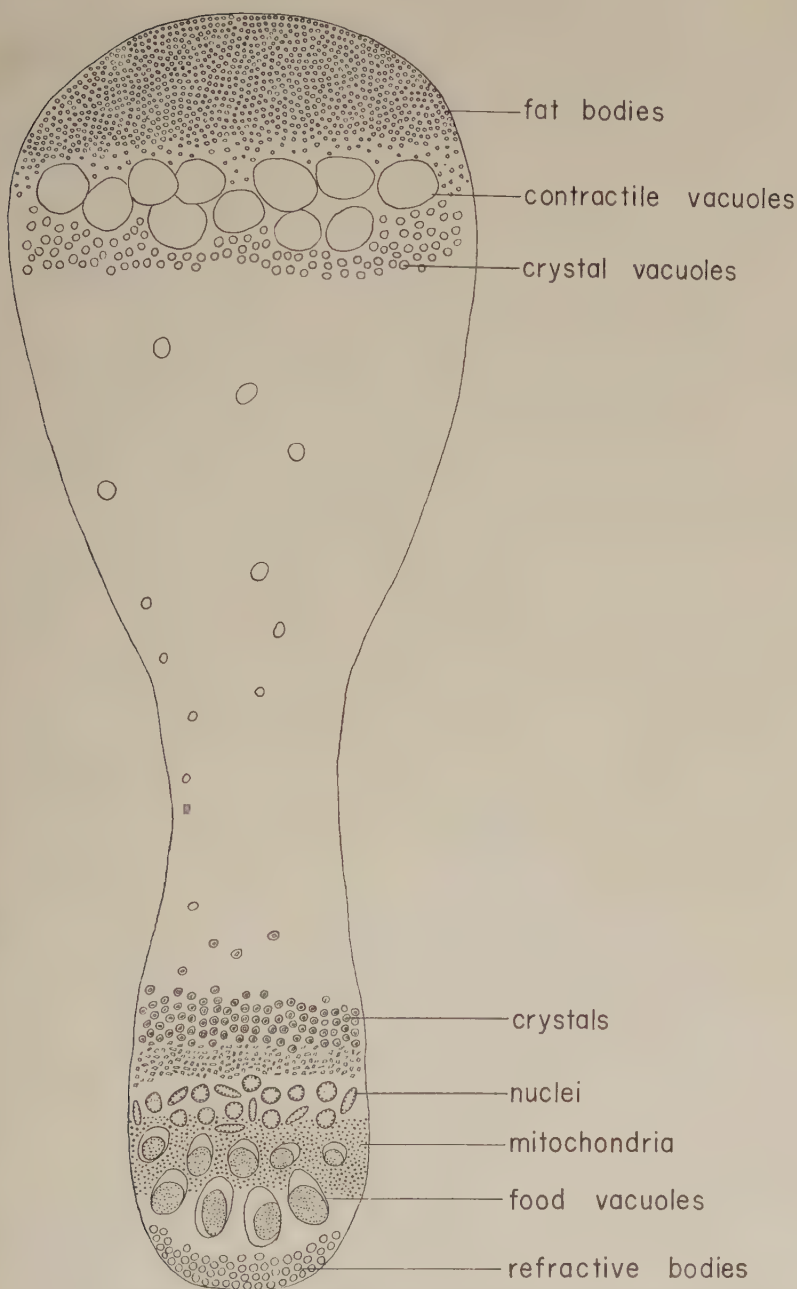


FIGURE 1. Diagrammatic sketch showing the stratification of cytoplasmic inclusions in *P. carolinensis* centrifuged for 45 min. at 740 g.

nucleus of *A. proteus*, but this does not occur in *P. carolinensis*, at least not at the forces employed. Neither Andresen (1942) nor Wilber (1945) reported nuclear stratification in *Pelomyxa*. In addition to the strata already noted, Andresen observed hyaline areas centripetal to the refractive bodies. At the forces employed in the present work, a clear area was occasionally observed centripetal to the refractive bodies, but a hyaline region was never observed between the crystal and contractile vacuoles at the centripetal pole.

The organisms respond favorably to centrifugation at a force of 700 g, and generally over 90 per cent of the animals survive with no apparent ill effects. Unless the individuals are maintained in ice water, redistribution of the cytoplasmic inclusions begins immediately upon removal from the centrifuge. Cytoplasmic redistribution begins with the formation of one or more broad pseudopodia at the centrifugal end. These pseudopodia are very rounded and contain only mitochondria. After the initial pseudopod formation, additional pseudopodia are projected from all areas of the body. Then there occurs a general streaming of the crystals, food vacuoles, nuclei, and mitochondria toward the centripetal pole. The fat droplets remain relatively stationary and clumped. During this process of redistribution, the organisms remain oriented along the centrifugal-centripetal axis, and no locomotion takes place.

Redistribution of the cytoplasmic granules is completed generally within 30 min. to 1 hour, and the organisms then proceed to show typical amoeboid movement. Even after this amount of time, however, the fat granules can be seen circulating en masse within certain individuals.

Vital staining with neutral red. Because of our interest in the Golgi bodies and, since many workers have considered neutral red to stain various aspects of the Golgi apparatus (MacLennan, 1941), it seemed relevant to subject the organisms to vital staining with neutral red. Andresen (1942, 1945) reported a neutral-red staining system of vacuoles in *Pelomyxa* and then extended his observations to *A. proteus* (1946).

At the concentration of neutral red employed (1:100,000 w/v) there appear to be no toxic effects due to the dye, and the organisms immediately settle and actively move on the bottom of the staining vessel.

After 30 min. in the staining solution, many of the food inclusions are stained a bright red, and the fluid within the food vacuoles is faintly orange. In addition to the food vacuoles, small inclusions located within vacuoles of varying dimensions become perceptible (FIGURE 2). These small granules vary in number for 1 to 60 within each vacuole and are stained a bright red. Many

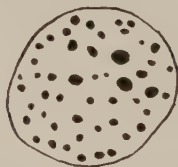


FIGURE 2. Camera lucida sketch of a neutral red vacuole formed after vital staining for 30 min. in neutral red (1:100,000). $\times 1860$.

of the granules are differentiated into 2 regions, a dark red cortical region surrounding an inner area of lighter color. At this time the granules are almost all between 0.5 to 1.5 μ in diameter. The vacuoles that contain them range from 4 to 12 μ in diameter, with the majority measuring approximately 8 μ . There seems to be no correlation between the size of the vacuoles and the number of granules within them; small vacuoles often contain more granules than larger ones. The granules undergo marked Brownian movement, indicating a vacuolar fluid of low viscosity. In contrast to the fluid within the food vacuoles, which becomes colored, the fluid within the neutral red vacuoles remains unstained. Both the vacuoles and their inclusions are preserved by chromic-osmium fixation.

After staining for 2 to 3 hours, the contents of nearly all the food vacuoles are stained red. Moreover, many of the crystal vacuoles concentrate the stain, and the crystals are apparent as unstained structures generally occupying an eccentric position within an orange-colored vacuole.

It is evident that after this period of time in the stain, the neutral red vacuoles have increased in both size and number. Vacuoles were measured ranging in size from 4 to 20 μ ; the majority were about 10 to 12 μ in diameter. The granules within the vacuoles vary between 0.5 to 5.5 μ in diameter. The larger granules are very similar in size and shape to the refractive bodies that are unstained in the cytoplasm. In certain instances, small, red bodies were found within large food vacuoles. In all respects, these structures are indistinguishable from neutral red bodies located in cytoplasmic vacuoles.

There is very little change in the gross morphology of the neutral red vacuoles until the organisms have been in the stain for 48 hours. At this time, in contrast to previous observations, the granules show a tendency to clump around the periphery of the vacuoles, and most of the granules accumulate in one area of the vacuole (FIGURES 3, 4, and 5). This gives the vacuole a centrifuged-like appearance. The vacuoles have increased slightly in size, ranging from 7 to 35 μ , with the majority around 15 μ in diameter.

Except for increases in the size of vacuoles, subsequent staining reveals no fundamental alterations from the previous description. One interesting observation was made on some organisms that were crushed after being in the staining solution for 72 hours. Each neutral red vacuole possessed a cap of refringent material on its surface (FIGURES 4, 5, and 6). Viewed from the side, the refringent caps are crescent-shaped, while in surface view they appear rounded with a diameter varying between 3 to 8 μ . Subsequent observations made on crushed organisms stained for shorter periods revealed that the refringent caps appeared on the surface of the neutral red vacuoles in animals stained for 2 hours or longer. The closest observation under bright field and phase microscopy could not reveal the origin or mechanism of formation of these structures, which were never observed on any other type of vacuole.

Organisms were observed intermittently after staining for periods as long as 6 days. After 6 days, vacuoles 50 μ in diameter are often encountered, while most of the vacuoles are 17 to 21 μ in diameter. The organisms are completely filled with these vacuoles, to the point that other cytoplasmic inclusions are

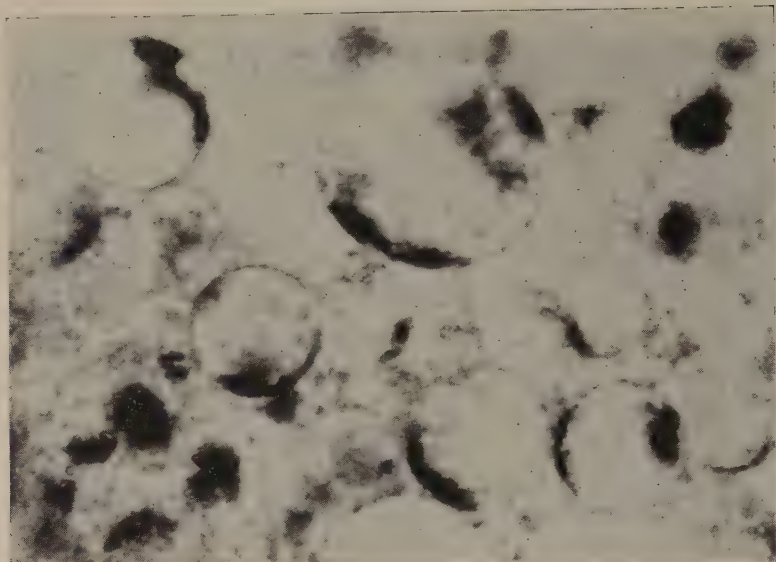


FIGURE 3. Photomicrograph of neutral red vacuoles in cytoplasm of organism vitally stained for 48 hours. The clumping of the neutral red granules on the periphery of the vacuoles is apparent. $\times 1000$.



FIGURE 4. Camera lucida sketch of a neutral red vacuole observed in an organism that was crushed after vital staining for 66 hours. A refractive cap is apparent on the vacuole surface. $\times 1860$.

difficult to see (FIGURE 3). The vacuoles are often clumped together in large masses. The granules are definitely arranged on the periphery of the vacuoles, and most of the granules are grouped at one side of the vacuole. Many of the granules appear to be fused together. No Brownian movement is observed within the crescentic area, although some $0.5\text{-}\mu$ granules show Brownian movement at other areas within the vacuole.

After six days in the staining solution, the organisms begin to show signs of degeneration, presumably due to the toxic effects of the dye. For these reasons, observations on the vitally stained organisms were discontinued at this time.

Centrifugation of vitally stained organisms. Animals stained vitally in neutral red for 36 hours were centrifuged at 700 g to determine how the neutral red vacuoles would stratify in relation to the other constituents. In addition to showing the relative position of these vacuoles, the other cytoplasmic constituents are clearly defined because of their varying affinities for the dye.

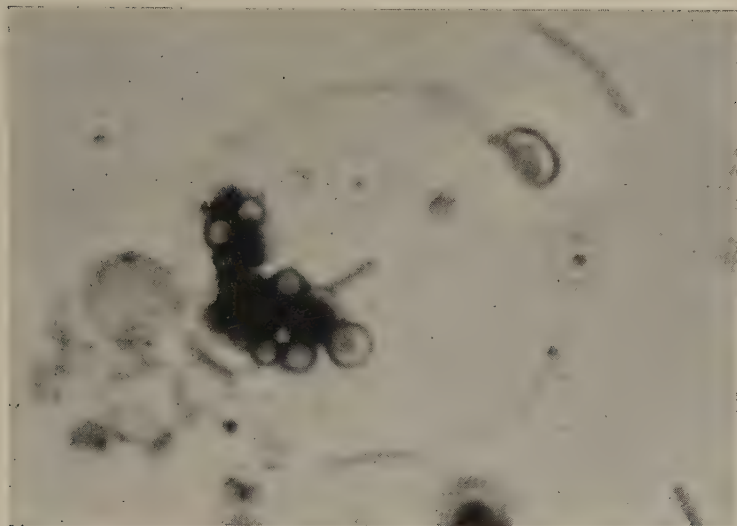


FIGURE 5. Photomicrograph showing, as in FIGURE 4, a neutral red vacuole observed in an organism that was crushed after vital staining for 72 hours. $\times 2000$.

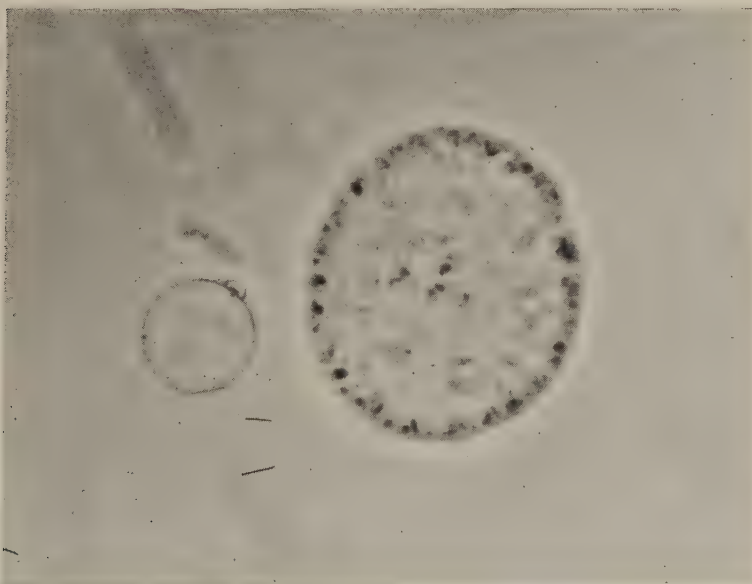


FIGURE 6. Photomicrograph of the refractive cap on a small neutral red vacuole observed in crushed organism after vital staining for 72 hours. The other structure is a nucleus. $\times 2000$.

At the centrifugal pole, the thin layer of refractive bodies varies in color from yellowish-orange to red. This is due to the great variation in stainability exhibited by the individual refractive bodies, since some are colored a deep red while others are completely colorless. The food vacuole and mitochondrial area appear deep red, almost black in color. Since the mitochondria are unstained, this coloration is due to the intense staining of the fluid and food particles within the food vacuoles. The nuclear region, together with the ejected crystal area, appears light red due, evidently, to the accumulation of alpha granules in this area. The crystal vacuoles are bright orange.

At the centripetal pole, the fat does not accept the stain and appears brownish. The layer of large vacuoles centrifugal to the fat is absolutely colorless and is the only completely colorless region in the centrifuged, vitally stained organism. The small vacuoles that accumulate centrifugal to the large vacuole area are bright orange in color, which suggests that they are empty crystal vacuoles.

Since the stratification of the neutral red vacuoles varies from organism to organism, only certain generalizations can be made. At the centrifugal forces employed, the vacuoles tend to accumulate in the centrifugal end directly centripetal to the crystals. In most organisms, only the larger vacuoles are found in this area, while the remaining vacuoles are distributed more or less uniformly in the region between the crystals and the centripetal pole. In some cases, however, the reverse situation was observed, and the larger vacuoles accumulated centripetally.

In the course of the centrifuge experiments a curious variation was noted in the distribution of the fat droplets. Organisms left in the stain for more than 48 hours revealed no characteristic fat layer upon centrifugation. Perhaps this was due to a breaking off of the fat layer during centrifuging, but the possibility that the visible fat droplets participated in neutral red vacuole formation was suggested. To determine the tenability of this hypothesis, animals were centrifuged, cut into light and heavy halves by means of microneedles, and placed within watch glasses containing neutral red in a concentration of 1:100,000. If the fat droplets participate in vacuole formation, the formation of vacuoles in the heavy end should be greatly reduced, since most, if not all, of the visible fat is located in the light half.

The results indicate that the presence of visible fat is not necessary for neutral red vacuole formation. Observations on heavy halves stained with neutral red do not differ significantly from results obtained on uncentrifuged animals. Within one half hour after being placed in the stain, the organisms possess neutral red bodies enclosed within vacuoles. The subsequent enlargement of the granules and growth of the vacuoles do not differ from the changes observed in uncentrifuged specimens. Survival of the heavy halves compares favorably with that of uncentrifuged organisms, indicating that the fat and empty vacuolar portions of the cytoplasm do not exert a protective influence against the toxicity of the dye. The light halves generally do not survive longer than 12 hours in the dye solution.

Golgi techniques. The presence of Golgi bodies has not been described in *Pelomyxa*, although they have been reported in other amoebae (Brown, 1930;

Mast and Doyle, 1935a, Causey, 1925; Hall, 1930; Das and Tewari, 1955). Accordingly, the animals were subjected to the classic silver and osmium techniques.

Of the various silver methods, Aoyama's technique was used most extensively in the present study, and the following description pertains to Aoyama material, although the other methods gave similar results. This material was silvered for 14, 18, or 40 hours, with no obvious differences in results. The results obtained were highly inconsistent and varied from organism to organism. Nuclei, food vacuoles, and refractive bodies were preserved, but the other cytoplasmic inclusions were generally not discernible. In some preparations, small vacuoles 3 to 5 μ in diameter were preserved, and these were interpreted as being crystal vacuoles.

The only structures that were blackened by the treatment were occasional inclusions in food vacuoles and small granules 0.5 μ in diameter. The granules are irregularly distributed in the cytoplasm, and often are arranged in small clumps. Other granules of similar size were occasionally observed on the inner margins of vacuoles that vary in size between 5 to 15 μ . Since these granules correspond to nothing observed in the living or fixed animal, they are interpreted as being localized precipitations of silver.

Contrary to the inconsistent results obtained with the silver methods, the osmium techniques give consistent and readily reproducible results. These techniques preserve all the cytoplasmic inclusions with the exception of the crystals and the alpha granules. The following description concerns animals fixed in chromic-osmium and postosmicated in 2 per cent osmium tetroxide for 8 days at 35° C. The results are typical of all the techniques.

Two kinds of inclusions are observed to be consistently blackened: (1) globules ranging from 1.5 to 4 μ in diameter; and (2) vacuoles ranging in size from 3 to 7 μ . The globules are uniformly darkened, while only the peripheral regions of the vacuoles are impregnated. The contents of food vacuoles are also intensely blackened, but the food vacuole membranes remain colorless (FIGURE 8). Mitochondria reduce the osmium somewhat, and appear greyish. Nasonov (1924) reported the darkening of the contractile vacuoles in *Vorticella* after prolonged osmification, and he homologized the contractile vacuole of Protozoa with the Golgi apparatus of Metazoa. His observations have been confirmed and extended by other workers (Gatenby *et al.*, 1955). In *Pelomyxa*, the contractile vacuoles, as identified by their mitochondrial covering, are definitely not blackened.

To obtain further information concerning the identity of the osmiophilic structures, organisms were stratified by centrifugation prior to fixation and osmification. After such treatment, the small globules are found at the centripetal pole, while the vacuolar structures are located in a wide region that extends from the centrifugal pole to a region just centripetal to the nuclei (FIGURE 7). While most of the vacuoles accumulate in the nuclear region, numerous vacuoles are always observed extending all the way to the centrifugal end. These results indicate that the small, darkened globules are definitely fat droplets, while the vacuolar structures are the crystal vacuoles. The accumulation of crystal vacuoles centrifugal to the nuclei is somewhat surprising, since



FIGURE 7. Photomicrograph of a 6- μ section of an organism that was centrifuged, fixed, and osmicated for 8 days at 35° C., showing accumulation of crystal vacuoles at centrifugal (lower) end and fat droplets at centripetal (upper) end. $\times 140$.

crystals were never observed to concentrate in this region in unstained, centrifuged organisms. Measurements were made on the vacuoles that accumulated centrifugal and centripetal to the nuclei, and no differences in size or morphology were detected.

On the basis of positive results obtained with silver and osmium techniques, Mast and Doyle (1935) reported that the cortical region of the refractive bodies was Golgi material. While the refractive bodies are preserved, it has been impossible to demonstrate a blackening of the cortical region with the techniques employed in the present study.

Discussion

Centrifuge studies. With regard to the stratification of the cytoplasmic inclusions of the Sarcodina by centrifugal force, previous workers (Mast and

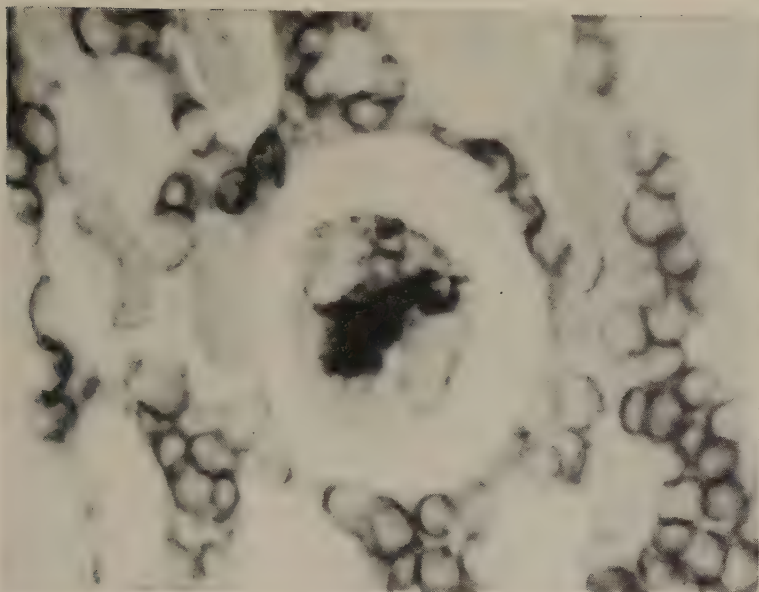


FIGURE 8. Photomicrograph of a 6- μ section of an organism treated as in figure 7, showing food vacuole surrounded by crystal vacuoles and some nuclei. Note the intense osmification of the crystal vacuoles while the food vacuole membrane is not darkened. $\times 1000$.

Doyle, 1935a; Singh, 1938; Andresen, 1942; Wilber, 1945) are in complete accord on several points. Thus, there is unanimous agreement that the refractive bodies are always located at the extreme centrifugal end, while fat and contractile vacuoles are located centripetally. However, there is apparent disagreement concerning the relative location of the mitochondria. Singh and Wilber located the mitochondria in a position centripetal to the crystals, while Mast and Doyle, and also Andresen, showed them to be centrifugal to the crystals.

The present observations support the work of Mast and Doyle and of Andresen, and are in disagreement with the observations of Singh and Wilber. Why the latter workers found the mitochondria centripetal to the crystals is difficult to explain. The most obvious interpretation would be that differences in the centrifugal forces employed produced the observed discrepancies. Mast and Doyle used forces as high as 3575 g, and Andresen centrifuged at 5680 g (my calculations from data in Andresen's paper). Neither Singh nor Wilber indicated the forces they employed. Since, in the present study, the mitochondria were displaced centrifugal to the crystals by a force of only 340 g, it seems unlikely that the discrepancies can be explained by differences in centrifugal force.

Neutral red bodies. Dilute solutions of neutral red stain the material within the food vacuoles, the fluid within the crystal vacuoles and, at times, the refractive bodies within the cytoplasm of *P. carolinensis*. In addition, a series of vacuoles containing variable numbers of granules, the "neutral red bodies," is

regularly elucidated. Of these various inclusions, only the latter are not normally seen in unstained organisms. The question then arises as to whether the neutral red bodies represent a normal cellular inclusion or whether they arise *de novo* in response to the dye. The "vacuome" idea has been extensively reviewed (MacLennan, 1941) and has received some support from previous workers on Sarcodina (Hall, 1930; Hall and Loefer, 1930; Nigrelli and Hall, 1930). Goldacre (1950, 1952) has related the uptake of neutral red in *A. proteus* to adsorption of the dye on unfolded protein molecules in actively streaming organisms. According to his model, as the proteins fold in the tail region of the organism, the dye is released and accumulates in the cytoplasm. Goldacre, however, differentiates between this diffuse accumulation of dye in the tail region and the staining of neutral red bodies, which he considers to be a precipitation effect in the cytoplasm.

While no definite conclusions can be drawn at present, it seems most probable that the neutral red bodies are induced and therefore do not represent a "vacuome" or any other cytoplasmic inclusion. There is some evidence for this point of view. Andresen (1942) noted that neutral red bodies formed in both the light and heavy halves of organisms that were centrifuged and then cut into halves. In the present study, it has been possible to preserve the neutral red bodies by fixation in chromic acid-osmium tetroxide, but the same treatment fails to reveal similar structures in organisms that were not vitally stained prior to fixation.

If the neutral red bodies are not preformed granules, what is their possible significance? While this question cannot be definitely answered, one possibility is suggested. These structures appear to be lipid in nature, and identical structures are formed in the presence of other vital dyes (unpublished data). Perhaps the formation of these structures represents a protective mechanism against the toxicity of the dye. Conceivably, the organisms might utilize lipid to form an insoluble complex with the dye, thereby effectively removing the dye from the cytoplasm. This could explain why neutral red bodies are formed only in dilute solutions of the dye, since at higher concentrations the organisms are killed before the bodies are formed.

Osmiophilic structures. On the basis of various techniques a Golgi apparatus has been described for *Amoeba proteus* (Brown, 1930; Mast and Doyle, 1935) and for *A. verrucosa* (Das and Tewari, 1955).

Brown identified small granules and spherules distributed at random in the cytoplasm. He described the spherules as having darkened rims that surrounded a colorless interior. Mast and Doyle, using silver and osmium techniques, concluded that the cortical region of the refractive bodies represented Golgi material. They considered the spherules described by Brown to be the refractive bodies. Das and Tewari identified small granules in *A. verrucosa* and, in addition, they described two kinds of vacuoles that possessed darkened rims. Their conclusion was that all three structures were Golgi material and that they represented various phases in the formation of the contractile vacuole.

Similar structures were identified in the present study but, due to observations made on centrifuged material, a different interpretation must be made

concerning their role in the economy of the cell. It seems beyond question that the darkened granules observed by previous workers actually are the fat droplets. Similarly, the vacuolar structures observed by Das and Tewari apparently are not primordia of the contractile vacuoles, but rather represent the crystal vacuoles. The spherules described by Brown could likewise have been crystal vacuoles. Darkening of the refractive bodies as reported by Mast and Doyle remains unconfirmed.

Recently, electron microscopists have identified within amoebae structures that apparently are similar to the Golgi apparatus described in vertebrates (Cohen, 1957; Pappas, 1959). Since the crystal vacuoles are also identified in these electron micrographs, the vacuoles cannot be considered Golgi material, even though they give positive results with the various osmium techniques. MacLennan (1940) has shown that the Golgi element cannot be identified on the basis of osmium reduction alone.

In conclusion, it is tempting to speculate on the functional significance of the crystals. It had been thought that the crystals originated within food vacuoles (Mast and Doyle, 1935*a*; Wilber, 1945) and that they functioned as a food reserve. However, Andresen and Holter (1945) have shown that in *P. carolinensis* the crystals do not decrease in numbers during starvation, and hence obviously do not serve as a source of nourishment. Moreover, these investigators claim that the crystals arise in the cytoplasm, and are found only incidentally in the food vacuoles as a result of coalescence (this contention is strengthened by the observation, in the present study, that the food vacuole membranes are never darkened by the osmium treatment that consistently blackens the crystal vacuoles). Frequent expulsion of crystals was also observed in starving individuals. These observations were confirmed and extended in work on *A. proteus* (Andresen, 1946).

The fact that crystals apparently originate *de novo* in the cytoplasm, do not decrease in numbers during starvation, and are frequently observed to be expelled in large masses suggests that the crystals are a metabolic waste product. If this is true, perhaps these structures represent an accessory mechanism for the excretion of nitrogen.

References

- ANDRESEN, N. 1942. Cytoplasmic components in the amoeba *Chaos chaos* Linne. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **24**: 139-184.
- ANDRESEN, N. 1945. Coalescence between vacuoles during vital staining with neutral red of *Chaos chaos* L. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **25**: 147-155.
- ANDRESEN, N. 1946. Cytoplasmic changes during starvation and during neutral red staining of the amoeba *Chaos diffluens* (*A. proteus*). Compt. rend. trav. Lab. Carlsberg, Sér. chim. **25**: 169-190.
- ANDRESEN, N. 1956. Cytological investigations on the giant amoeba *Chaos chaos* L. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **29**: 435-555.
- ANDRESEN, N. & H. HOLTER. 1945. Cytoplasmic changes during starvation of the amoeba *Chaos chaos* L. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **25**: 107-146.
- BAKER, J. R. 1951. Cytological Technique. 3rd ed. Methuen. London, England.
- BROWN, V. E. 1930. The Golgi apparatus of *Amoeba proteus*. Biol. Bull. **30**: 240-246.
- CAUSEY, D. 1925. Mitochondria and Golgi bodies in *Endamoeba gingivalis* (Gros) Brumpt. Univ. Calif. Publ. Zool. **28**: 1-18.
- COHEN, A. I. 1957. Electron microscopic observations of *Amoeba proteus* in growth and inanition. J. Biophys. Biochem. Cytol. **3**: 859-865.

- DAS, S. M. & H. B. TEWARI. 1955. Golgi apparatus in *Amoeba verrucosa* Ehrenberg. *Current Sci.* **24**: 58-59.
- GATENBY, J. B., A. J. DALTON & M. D. FELIX. 1955. The contractile vacuole of Parazoa and Protozoa, and the Golgi apparatus. *Nature*. **176**: 301-302.
- GOLDACRE, R. J. 1952. The folding and unfolding of protein molecules as a basis of osmotic work. *Intern. Rev. Cytol.* **1**: 135-164.
- GOLDACRE, R. J. & I. J. LORCH. 1950. Folding and unfolding of protein molecules in relation to cytoplasmic streaming, amoeboid movement and osmotic work. *Nature*. **166**: 497-501.
- HALL, R. P. 1930. Cytoplasmic inclusions of *Trichamoeba* and their reaction to vital dyes and to osmic and silver impregnation. *J. Morphol.* **49**: 139-152.
- HALL, R. P. & J. B. LOEFER. 1930. Studies on Euglypha. I. Cytoplasmic inclusions of *Euglypha alveolata*. *Arch. Protistenk.* **72**: 365-376.
- KUDO, R. R. 1946. *Pelomyxa carolinensis* Wilson. I. General observation on the Illinois stock. *J. Morphol.* **78**: 317-352.
- KUDO, R. R. 1947. *Pelomyxa carolinensis* Wilson. II. Nuclear division and plasmotomy. *J. Morphol.* **80**: 93-144.
- MACLENNAN, R. F. 1940. A quantitative study of osmic acid impregnation in Protozoa. *Trans. Am. Microscop. Soc.* **59**: 149-159.
- MACLENNAN, R. F. 1941. Cytoplasmic inclusions. In *Protozoa in Biological Research*. : 111-190. G. N. Calkins, Ed. Columbia Univ. Press. New York, N. Y.
- MAST, S. O. & W. L. DOYLE. 1935. Structure, origin and function of cytoplasmic constituents in *Amoeba proteus*. I. Structure. *Arch. Protistenk.* **86**: 155-180.
- MAST, S. O. & W. L. DOYLE. 1935a. Structure, origin, and function of cytoplasmic constituents in *Amoeba proteus* with special reference to mitochondria and Golgi substance. II. Origin and function based on experimental evidence; effect of centrifuging on *Amoeba proteus*. *Arch. Protistenk.* **86**: 278-306.
- MCCLUNG, C. E., R. T. HANCE & E. V. COWDRY. 1950. Methods of cytology. In *McClung's Handbook of Microscopical Technique*. : 115-152. R. M. Jones, Ed. Hoeber. New York, N. Y.
- NASSONOV, D. 1924. Der Exkretionsapparat (kontraktile Vacuole) der Protozoa als Homologen des Golgischen Apparats der Metazoozellen. *Arch. mikroskop. Anat. u. Entwicklungsmech.* **103**: 437-482.
- NIGRELLI, R. F. & R. P. HALL. 1930. Osmiophilic and neutral-red-stainable inclusions of *Arcella*. *Trans. Am. Microscop. Soc.* **49**: 18-25.
- PAPPAS, G. D. 1959. Electron microscope studies on Amoebae. *Ann. N. Y. Acad. Sci.* **78**(a): 448.
- SINGH, B. N. 1938. The cytology of *Amoeba proteus* 'Y' and the effects of large and small centrifugal forces. *Quart. J. Microscop. Sci.* **80**: 601-635.
- TORCH, R. 1955. Cytological observations on *Pelomyxa carolinensis* with special reference to the mitochondria. *J. Protozool.* **2**: 167-177.
- WILBER, C. G. 1942. The cytology of *Pelomyxa carolinensis*. *Trans. Am. Microscop. Soc.* **61**: 227-235.
- WILBER, C. G. 1945. Origin and function of the protoplasmic constituents in *Pelomyxa carolinensis*. *Biol. Bull.* **88**: 207-219.

PARTICULATES OF AMOEBAE*

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Cytoplasmic inclusions or particulates may be classified in several ways, including size. Thus we find microscopic particulates, as well as submicroscopic structures, the latter ranging in size from 0.06 to 0.2 μ , being smaller than the theoretical limit of resolution of the light microscope. Information based on electron micrographs of thin sections, high speed centrifugal fractionation of homogenates, chromatographic separation, and surface chemical properties of proteins, points to the existence of a submicroscope particulate structure in cytoplasm (Kopac, 1950).

Although the evidence is not conclusive, most cytologists and cytochemists are of the opinion that the particulate components of cytoplasm are centers of enzyme activities and account for structural organization. Kopac (1951) suggested that submicroscopic particulates with a capacity of undergoing fibrillization may provide the basis for the sol-gel changes involved in amoeboid movement and cytokinesis. It is at this level that the essential physicochemical transformations occur, and here we must seek an understanding of the fundamental morphological patterns responsible for physiological activities (Lazarow, 1945; de Robertis *et al.*, 1948).

The empirical choice of media used for the fractionation and isolation of cellular particulates, such as nuclei and mitochondria, led to a series of experimental attempts to evaluate the effects of various media on the cytoplasm of amoeba. Previous reports on the evaluation of fractionation media (Kassel and Kopac, 1953) were based on changes induced in the microscopic particulates following the injection of various substances into the cytoplasm of *Amoeba proteus* and *Pelomyxa carolinensis*. This paper deals with a more critical analysis of the effects of these media on the submicroscopic particulates immediately following cytolysis, as determined by the use of drop-retraction and surface chemical techniques (Kopac, 1950).

Materials and methods. The techniques of culturing *Amoeba proteus* and *Pelomyxa carolinensis*, of microinjection, and the preparation of special solutions, have been previously described (Kassel and Kopac, 1953).

The standard Chambers' microinjection assembly was modified for drop-retraction measurements by including a micrometric screw syringe (MSS-3). This syringe, which has a capacity of 3 ml., has a macro control piston that can be manipulated by hand and, in addition, a threaded screw collar for delicately controlling the piston—a feature that is required for drop-retraction measurements. The special syringe is connected to the injection circuit by way of a rubber to a Luer-Lok adaptor.

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The micropipettes were prepared from capillary stock (outside diameter 0.85 mm.) of either soft, Pyrex, Jena one-red stripe, or Kimble glass, using a microburner as described by Chambers and Kopac (1950). The Barber type micropipettes, having small, fire-polished openings, are obtained when the glass, in a molten state, parts with a gentle snap. These fire-polished pipettes with a diameter of 5 to $10\ \mu$ at the orifice, were used in all the drop-retraction measurements reported here.

In addition, several specialized techniques were utilized during these investigations. The general procedures in each will be discussed here, but for the sake of clarity specific changes made will be described along with the results of experiments involving such modifications.

One of these procedures brings about the intracellular stratification, by centrifugation, of cytoplasmic particulates in accordance with their densities. The pelomyxas, after separation from other protozoa, were suspended in a density gradient consisting of a 2 per cent solution of soluble starch in distilled water. Preliminary centrifugation (International Centrifuge, type SB 1) at 350 g for 3 min., caused retraction of most pseudopodia; subsequent centrifugation at 2500 g for 20 min. produced complete stratification similar to that described by Andresen (1941-1943). On removal from the centrifuge, the tubes containing the pelomyxae were placed in a freezing-brine mixture to slow down the cytoplasmic streaming that rapidly brings about the redistribution of the cytoplasmic particulates. The preparation of fractions of centrifuged pelomyxae will be described in a later section.

One of the sensitive methods developed for measuring the surface chemical properties of cytoplasmic and other proteins is the drop-retraction technique (Kopac, 1943; 1950). This is a microadaptation of the surface chemical procedure originally developed by Devaux for studying monolayers of proteins at liquid-liquid interfaces. With the drop-retraction apparatus, the surface area of an oil drop, poised on the tip of a micropipette, can be increased or decreased by enlarging or reducing its diameter.

With the oil drop immersed in water or in salt solutions, the drop retains a spherical shape until it is completely retracted following the reduction of pressure within the drop retractor. A strikingly different situation develops if the oil first comes in contact with an aqueous medium containing proteins. As the drop is retracted by reducing the back pressure, the sphericity of the drop is maintained to a certain size, the critical diameter. If the drop is made smaller, the surface becomes crinkled, thereby producing the Devaux effect.

The following are some of the quantitative features of the drop-retraction method as applied to proteins. An oil drop of diameter D , in contact with the aqueous phase, presents an interfacial area of πD^2 at which protein molecules may become surface denatured. This oil drop, at any time, can be retracted to the point at which crinkling would occur, providing the drop were further reduced in size. As the drop is slowly retracted and as the critical diameter is approached, there will be a striking tendency for the poised drop to quiver. The diameter of the oil drop at the instant of quivering is taken as the critical diameter, d .

The critical interfacial area s equals πd^2 , and this represents the minimum area required by the proteins that are trapped at the oil-water interface and that cannot be driven out by the application of surface pressures (in this instance by reducing still further the interfacial area). Since the maximum area available for protein molecules is the area presented by the oil drop of diameter D , it is designated by A (equals πD^2). A convenient means of indicating the extent of surface denaturation of protein molecules is by the fraction s/A that equals $\pi d^2/\pi D^2$ (Kopac, 1950).

An oil drop may thus be expanded at the tip of micropipette and its diameter, D , is measured with a micrometer ocular. The instant of full expansion of the drop is taken as zero time and, at any selected time interval, the drop may be retracted to the critical diameter, d . As soon as the critical diameter is measured, the drop is re-expanded to its original diameter, D . At another selected time interval, the drop is again retracted and its critical diameter is measured. This process may be repeated as many times as required. The s/A values corresponding to the times at which the d values are measured are then calculated.

If the values of s/A are plotted against time, a parabolic curve is obtained ($s/A = kt^a$). A straight line results on plotting $\log s/A$ against \log time ($\log s/A = \log k + a \log t$). Kopac (1950) has used the slope of this straight line, a , and its intercept at 1 min., $\log k$, to compare the effects of various chemical agents on the surface denaturation of model protein systems, such as, tobacco mosaic virus, bovine plasma albumin, and ribonuclease. The application of this technique to the study of proteins released on cytolysis of an amoeba or *Pelomyxa* will be discussed in this report.

Results. The adaptation of the drop-retraction techniques to proteins released from cytolysed single cells presented a difficult problem: namely, to have the zero time for drop retraction correspond to the instant of cytolysis. The following procedure was developed in order to achieve this goal: amoebae were removed from the culture medium and washed three times with fresh Brandwein's solution (*Pelomyxa* were washed in distilled water). One amoeba was then transferred, with approximately 5 μ l. of Brandwein's solution, to a depression slide containing 150 μ l. of the testing medium. The amoeba was then transferred with 40 μ l. of the test medium and placed on a cover glass.

The amoebae were cytolysed by rapidly injecting the testing medium in sufficient quantity to cause rupture of the surface layer and extrusion of the cytoplasmic constituents. Prior to cytolysis, a drop of oil consisting of 1 part oleic acid in 100 parts of mineral oil, approximately 160 μ in diameter, was expanded on the tip of a fire-polished micropipette adjacent to the amoeba. The instant of cytolysis was taken as the zero time for the drop-retraction measurements, and the oil drop was immediately moved into the center of the cytolysed residue. The critical diameters were measured at 1, 3, 5, 7, and 9 min. after cytolysis. A fresh drop was then introduced near the site of the first drop, and measurements were made at the same intervals. The s/A values were calculated from the data and plotted against time on log-log paper (FIGURE 1).

The graphs of $\log s/A$ against \log time frequently showed two slopes. The

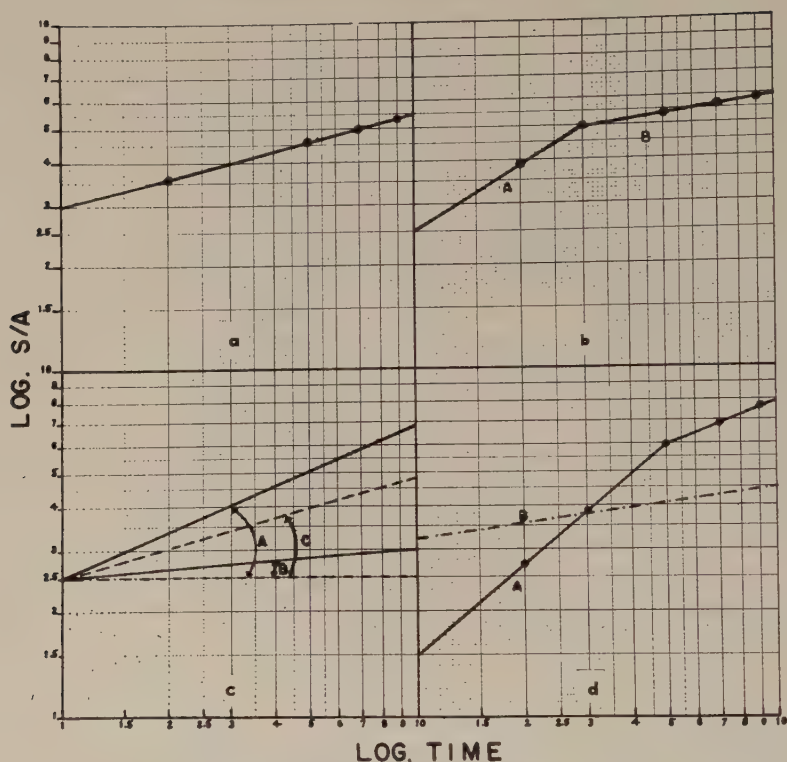


FIGURE 1. (a) An example of the typical straight line curve obtained from drop-retraction studies on homogeneous protein solutions (after Kopac). Such curves result from the plotting of $\log s/A$ (on the vertical axis) against \log time (on the horizontal axis). The value k is equal to the $\log s/A$ at 1 min. and the value a equals the slope of the curve. Both of these values are important in comparing the data obtained from studies conducted on various protein solutions, or under differing interfacial conditions. (b) A diphasic curve of the type obtained from drop-retraction studies of the proteins released following the cytolysis of a single amoeba or *Pelomyxa*. (c) Method of analysis of the diphasic, cytoplasmic protein curve (for detailed description see text). Angle C, the disaggregation angle, is assumed to be an indication of the rate of disintegration of the submicroscopic spherical aggregates, and is obtained by subtracting the secondary slope B from the primary slope A. In TABLES 1 and 2 these angles are expressed as the tangents of the angles involved. The extension of the labile period, by variations in the medium, led to a more gradual transition (as in media containing protamine) from the labile period to the establishment of uniform conditions. Under such conditions, the initial slope was also compared with the secondary slope obtained 50 or 60 min. after cytolysis. This procedure is indicated along with the results, when used. (d) An example of the typical curve obtained following the cytolysis of a single amoeba or *Pelomyxa* in a solution containing protamine 2 mg./cc. in physiological salt solution. Curve A, from studies during the first 10 min. following cytolysis; curve B, from studies conducted 50 min. following cytolysis. A composite of A and B resembles the two-slope curve obtained in b.

initial slope was steep, while the second slope resembled that of an ordinary protein system (Kopac, 1948; 1950). The latter slope, on the basis of model measurements, probably indicates the tendency of the proteins to undergo surface denaturation. The initial slope, in all probability, indicates surface de-

nuration plus some unusual augmentation factor. One possibility is that this slope indicates the breakdown of protein aggregates (or, concurrently, the rather rapid release of proteins susceptible to surface denaturation) that thus supply increasing amounts of low molecular weight proteins that become surface denatured at the earlier times and also during the times represented by the second slope (FIGURES 1 and 2).

As a first approximation, we have considered that the initial slope represents a combination of protein release and surface denaturation, and that the second slope is primarily surface denaturation. Accordingly, the difference between

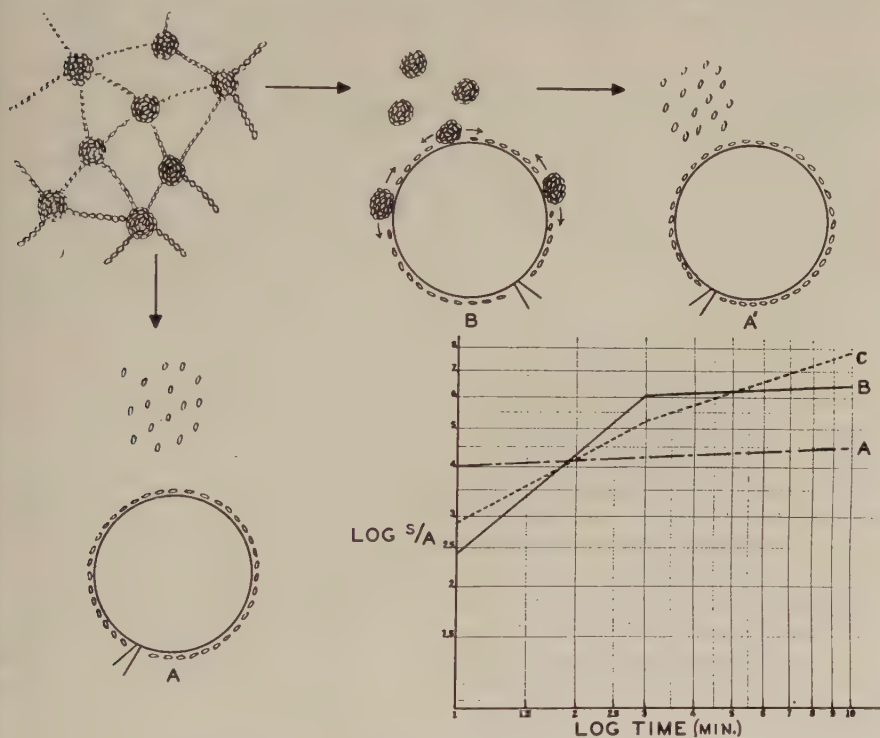


FIGURE 2. Probable nature of post-cytolytic disintegration.

In a nonphysiological solution, the submicroscopic aggregates (upper left) disintegrate, immediately following cytolysis, to release their low molecular weight protein components. These proteins saturate the oil-water interface (as in A, lower left) and, on unfolding, produce a protein denaturation curve that resembles that of a homogeneous protein solution (A in graph).

In a more physiological solution, many of the aggregates are maintained (B) and then gradually disintegrate at the interface to release their low molecular weight proteins (A'). In the resulting diphasic curve (B and C in graph), the slope during the first 3 min. is assumed to be the result of the gradual disintegration of the aggregates (as in B); the slope from 3 to 10 min., as a result of the unfolding of proteins derived from disintegrated aggregates (as in A'). If the aggregates are disintegrated before the oil-water interface is introduced (as in A), then the slope of the curve will be essentially similar to that of A in the graph.

Where the media are nonphysiological, the oil-water interface must be introduced immediately after cytolysis in order to get a type B effect, otherwise the effect will be essentially type A.

TABLE 1
ANALYSIS OF DROP-RETRACTION CURVES
Average Slopes (a values) at $t = 10$ min.

Medium and fraction cytolized	Initial ($\tan \angle A$)	Second ($\tan \angle B$)	Difference ($\tan \angle C$)	Log k at 10 sec.	Time to reach uniformity
Distilled water:					
Entire <i>Pelomyxa</i>	0.160	0.131	0.026	0.322	3 min.
Light fraction	0.383	0.190	0.179	0.126	5 min.
Heavy fraction	0.140	0.140	0.0	0.219	<1 min.
Cytoplasmic salt solution:					
Entire <i>Pelomyxa</i>	0.373	0.140	0.221	0.291	10 min.
Light fraction	0.449	0.154	0.275	0.162	10 min.
Heavy fraction	0.198	0.108	0.087	0.245	<1 min.
Protamine:					
Entire <i>Pelomyxa</i>	0.692	0.593	0.398*	0.072	50 to 60 min.
			0.069†		
Light fraction	0.778	0.522	0.464*	0.035	50 to 60 min.
			0.181†		
Heavy fraction	0.309	0.309	0.0	0.053	<1 min.
Sucrose (0.88 M):					
Light fraction	0.283	0.205	0.073	0.104	<1 min.

* Tangent $\angle C$ derived from comparison with stable slope attained 50 to 60 min. after cytolysis.

† Tangent $\angle C$ derived from comparison with slope 10 min. after cytolysis.

these two slopes (designated as $\angle C$) may be taken as a rough measure of the breakdown of protein containing structures, for example, submicroscopic aggregates or particulates. The data for these slopes are given in TABLE 1.

The work with *A. proteus* raised questions about (1) the effects of diffusion of proteins away from the principal zone of cytolytic residue, and (2) the loss of proteins adsorbed on the surface of preceding, and succeeding, oil drops.

The question of diffusion was approached by cytolizing an amoeba and introducing an oil drop at various distances away from the zone of residue. For example, an oil drop was placed approximately 800 μ from the cytolytic residue, and retracted after 1 min.; a fresh drop was introduced 5 min. after cytolysis and again retracted after 1 min. of exposure. This process was repeated every 5 min. and, even after 30 min. had elapsed after cytolysis, no amount of surface denatured protein could be detected. In a similar experiment, oil drops were approximately 500 μ from the cytolytic residue. Expansion and retraction of drops were repeated as described above and, even after 25 min., no protein could be demonstrated at the oil-water interface. In a third series, an oil drop was placed at 175 μ from the residue, and within 10 min. the s/A value was 0.03, which indicates only a trace amount of surface-denatured protein. Accordingly, loss of proteins by diffusion was not significant.

The problem of protein depletion due to the adsorption and removal from the medium by successive oil drops was approached by cytolizing an amoeba and introducing oil drops at selected times after cytolysis. The results from this procedure were compared with a drop exposed during the similar period in the same medium in which several drops had previously been exposed; namely, a drop exposed at 30 min. after cytolysis was compared with the third drop in

a series done in the same medium. In each instance, the results were almost identical in both the slope and amount of protein adsorbed and surface denatured.

The possibility was still present that the microscopic cytoplasmic particulates such as, crystals, vacuoles, and refractile bodies, were contributing to the amount of surface-denatured protein that could be detected at the oil-water interface. In order to settle this possibility and to evaluate the role of the larger particulates, it was necessary to turn to *Pelomyxa*, which could be centrifuged to stratify its cytoplasmic particulates and which, because of its large size, could be cut to provide fractions large enough for drop-retraction measurements following cytolysis.

The *Pelomyxa*, after the particulates were stratified by centrifugation, were cut into "light" and "heavy" fractions with glass needles under a dissecting microscope. The light and heavy fractions were separated and recentrifuged at 2500 g for 15 min. These were again cut, thus providing 4 fractions: (1) a light fraction containing mainly the clear matrix; (2) a light fraction containing matrix and mitochondria; (3) a light-heavy fraction containing matrix and various denser granules; and (4) a heavy fraction containing nuclei, food vacuoles, granules, and only small amounts of matrix.

The physical appearance and other properties created the impression that the light fractions were weak and friable, while the heavy fractions were dense and resistant to mechanical treatment. At the time of cytolysis, however, the opposite proved to be true. The light half was tough, rubbery in consistency, and resistant to cytolysis. The heavy half, on the other hand, was easily penetrated with a micropipette, friable, and offered little or no resistance to cytolysis. The light fractions were actively motile and formed reasonably typical pseudopodia. The heavy fractions remained rounded and rather quiescent. These fractions were subjected to cytolysis and the drop-retraction measurements were made as already described. The results are summarized in TABLE 1.

In addition to the above, several whole *Pelomyxa* were first injected with sodium thymonucleate at 0.075 mg./cc. and then cytolized in physiological salt solution. A second group of *Pelomyxa* was injected with sodium thymonucleate at 0.075 mg./cc. and then cytolized in the protamine medium. The results of drop-retraction studies of these cytolized *Pelomyxa* are tabulated in TABLE 2.

TABLE 2
ANALYSIS OF DROP-RETRACTION CURVES
Average Slopes (a values) at $t = 10$ min.

Medium and fraction cytolized	Initial ($\tan \angle A$)	Second ($\tan \angle B$)	Difference ($\tan \angle C$)	Log k at 10 sec.	Time to reach uniformity
Entire <i>Pelomyxa</i> :					
Cytoplasmic salt soln.	0.373	0.140	0.221	0.219	10 min.
Cytoplasmic salt soln. (DNA)	0.264	0.190	0.069	0.305	3 min.
Protamine	0.692	0.593	0.398	0.072	50 to 60 min.
Protamine (DNA)	1.046	0.464	0.392	0.024	50 min.

Drop-retraction studies of light halves cytolized in the iodide, nitrate, acetate, and bromide modifications of the physiological salt solution indicated that the disaggregation angles (Angle *C*) could be arranged, in order of increasing magnitude, that is, greatest for bromide, least for iodide. The labile period was short in each instance, and in several experiments with acetate a spontaneous Devaux effect was observed within 1 min. after cytolysis.

Discussion

The drop-retraction technique thus provides a sensitive method for studying the effects of fractionation media upon submicroscopic structures. At this level of organization we cannot depend upon the more gross observations usually utilized in microscopic studies. Here we must deal with the more subtle changes occurring in a highly labile system. The drop-retraction technique begins to give us some information as to what happens to the labile proteins of the cytoplasm at the time of cytolysis. As regards the interpretation of drop-retraction studies on cytoplasmic proteins, we must depend to a great extent on the information obtained by Kopac (1950) from the extensive studies of homogeneous protein systems, including over 60 different organic phases and proteins, namely: bovine and human plasma albumins, thrombin, crystalline trypsin and chymotrypsin, crystalline ribonuclease, hyaluronidase, protamine, protamine-nucleate complexes, liver nucleoproteins, thymus nucleohistone, and tobacco virus nucleohistone. Previously, Kopac (1948) had also investigated the action of various chemical agents that either enhance or inhibit the surface denaturation of proteins. For homogeneous protein systems Kopac (1948) found the relationship between $\log s/A$ and \log time to be linear. The slope of this line, a , was interpreted as indicating the rate of protein denaturation, the s/A value indicating the amount of protein present per unit area at the oil-water interface. The intercept at 1 min., k , and the slope a were used as the basis for comparing the surface denaturation of proteins under various interfacial conditions. Kopac's results (1950) indicate the following: (1) in some instances, k may actually represent the initial adsorption and preliminary unfolding of proteins at the interface, while the slope a is a measure of the rate of unfolding of those molecules originally adsorbed; (2) where k is high, the slope may not be a true indication of the rate of unfolding, due to the space difficulties involved when the interface is crowded; and (3) with low k values and with high a values the situation may be complex, the low k value indicating that both the number of the molecules and their degree of unfolding is low. The high values of the slope a may be due to an increase in the number of adsorbed molecules, an increase in the degree of unfolding of the molecules initially adsorbed, or a combination of both.

Refinements in the technique based on preliminary investigations on diffusion, loss of protein adherent to the oil drop, and the role of microscopically visible inclusions led to procedures that gave consistent results, so that none of these possible 3 sources of error was felt to play any significant role. The data obtained with fractions of *Pelomyxa* indicated rather conclusively that the cytoplasmic matrix is the source of the protein adsorbed at the oil-media interfaces. The results obtained following cytolysis in a medium containing the physiologi-

cal salt solution (KCl 0.042 M, NaCl 0.017 M, CaCl_2 0.0015 M, MnCl_2 0.001 M) plus protamine appear to present just such a complex system as described by Kopac (1950) as shown in FIGURE 1. The initial values for k , extrapolated to 10 sec., were low (of the order of 0.035) and the slopes comparatively high. The difference between the initial slope and the slope obtained later is designated as the c value, and these values were much higher following the addition of protamine than those with other media.

On the other hand, cytolysis in a nonphysiological medium would provide a large initial amount of protein of low molecular weight to be acted upon by surface forces (FIGURE 2). The k value at 10 sec. under such conditions would be high; the slope and corresponding c value (disaggregation effect), low. This appeared to be the situation with media such as the physiological salt solution containing DNA at 2 mg./cc., the substituted nitrate, iodide, bromide, and acetate salt solutions, or distilled water. Thus, the chloride salt solution apparently enhanced the stability of the aggregates, yielding lower k values and higher disaggregation angles than those obtained above. Moreover, the addition of protamine to the medium resulted in still lower k and higher c values.

In most of the other media tested, the initial period of lability as indicated by low k values and high slopes disappeared in about 5 to 10 min. The subsequent introduction of oil drops produced high k values and low slopes typical for low molecular weight proteins. It was assumed that during this period the aggregates of protein had broken down, leaving only the more stable, low molecular proteins. Apparently this initial disaggregation is delayed in a medium containing protamine. The delay is temporary, however, and the aggregates slowly break down to provide a reserve of proteins that become surface denatured on the introduction of an oil-water interface. The break down of aggregates is independent of surface forces, since high k values and low slopes are obtained at any time within this period on introduction of an oil-water interface.

The injection of DNA into the living *Pelomyxa*, followed by its cytolysis in a protamine solution, yielded data comparable to those obtained with protamine alone. This again may be taken as an indication that DNA is capable of protecting the spherical aggregates in the intact cell in some manner, thus rendering them initially more stable on cytolysis. The differences between the k and c values obtained from *Pelomyxa* cytolized in media containing protamine and from *Pelomyxa* cytolized in the same medium but preceded by the injection of DNA are not clear-cut at this time. However, they do offer an indication that the further investigation of protamine-nucleate complexes may prove profitable.

In aqueous media containing proteins of low molecular weight, the introduction of oil drops at various times yields identical curves. These results are possible only if the amount of protein available for surface denaturation remains constant, while the rate of unfolding, as indicated by the slopes, is a function of the forces acting at the interface.

Such uniformity in concentration of cytoplasmic proteins released on cytolysis apparently does not exist. Cytoplasmic proteins in intact protoplasm are resistant to surface denaturation (Kopac, 1950) and, at the instant of cytolysis,

no appreciable amounts of proteins susceptible to surface denaturation are available. Following cytolysis there is a rapidly increasing availability of proteins susceptible to surface denaturation until, at some time following cytolysis, the concentration of such proteins reaches a constant level. At this time the surface denaturation effects are similar to those obtained at any time with proteins of low molecular weight.

The importance of time in relation to cytolysis is emphasized by the observations that, with many media, uniform conditions are established within 5 to 10 min. after cytolysis. This would indicate that the break down of protein aggregates occurs rapidly in such media, indicating thereby a short labile period.

At present the homogenizing procedures and subsequent fractionation procedures used in many biochemical studies may be expected to extend well beyond the labile period. The importance of the time factor in relation to homogenization procedures cannot be emphasized too strongly, since most of these techniques involve long periods of homogenization and fractional centrifugation before the isolated material is studied. In view of the possibilities of the twin problems of disintegration of the submicroscopic aggregates and their adsorption or agglutination, materials obtained after uniform conditions have been established may in no way resemble their native cytoplasmic components. It is quite possible that the rapid establishment of this uniformity may account for the exact reproducibility of results, which has frequently been used as an argument in favor of the reliability of data obtained from fractionation procedures. Thus the assignment of specific properties, such as enzyme systems, to specific cytoplasmic components may be completely misleading. The observation that enzyme systems are always associated with specific isolated fractions does not eliminate the possibility that this association may have developed as the result of disintegration, agglutination, or both.

Although additional studies of media containing protamine are planned at present, it has been established that the labile period can be extended to approximately one hour. In an ideal medium, it should be possible to obtain the diphasic curve at any time following cytolysis providing the protein aggregates are prevented from disintegrating. Substances such as nucleic acids, or protamine, or combinations of the two temporarily satisfy the requirements of an adequate medium by their ability to prolong the labile period. Other substances, yet to be tested, may prolong this period even more. In all probability no single medium will maintain all the fractions of cytoplasm equally, and it may be necessary to develop specific media to meet the demands of the individual cytoplasmic components. The drop-retraction technique provides the only known method of determining the existence of submicroscopic aggregates of proteins derived from the cytoplasmic matrix, and for testing the stability of these aggregates in various fractionation media.

Summary

The drop-retraction technique has been utilized in a study of cytoplasmic proteins immediately following cytolysis.

As indicated by drop-retraction measurements, cytolysis is followed by a short period of lability during which drastic changes occur in the submicroscopic

organization of cytoplasm. The short duration of this labile period raises some questions regarding the relationship between native constituents of cytoplasm and those isolated by homogenization and fractionation procedures, since it is probable that the cytoplasmic components have undergone striking alterations during the procedures.

The addition of protamine at concentrations of 2 mg./cc. to the physiological salt solution increases the labile period to approximately 60 min. Preliminary results indicate that protamine-nucleate complexes may extend the labile period still further.

It is postulated that, in nonphysiological media, the submicroscopic aggregates disintegrate immediately following cytolysis to release proteins that have properties resembling typical proteins of low molecular weight. These proteins, on reaching an experimentally introduced oil-water interface, unfold and produce a surface denaturation curve resembling that of a medium containing a protein of low molecular weight.

In a more physiological medium many of the aggregates are maintained for a while and then gradually disintegrate. On reaching an oil-water interface these aggregates disintegrate, release their proteins, and produce a diphasic surface denaturation curve. The first portion of the diphasic curve is assumed to indicate the disintegration of the aggregates, while the second portion indicates the unfolding of low molecular weight proteins derived from the aggregates. If the aggregates disintegrate before reaching an oil-water interface, the resulting surface denaturation curve is essentially similar to that obtained with a medium containing proteins of low molecular weight.

Acknowledgment

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References

- ANDRESEN, N. 1941-1943. Cytoplasmic components in the amoeba *Chaos chaos* Linne. Compt rend. trav. Lab. Carlsberg Sér. chim. **24**: 140-180.
- CHAMBERS, R. & M. J. KOPAC. 1950. Micrurgical technique for the study of cellular phenomena. In McClung's Handbook of Microscopical Technics. : 492-543. 3rd ed. Hoeber. New York, N. Y.
- DE ROBERTIS, E. D. P., W. W. NOWINSKI & F. A. SAEZ. 1948. General Cytology. Chap. IV. : 63-88. Saunders. Philadelphia, Pa.
- FERRY, J. D. 1948. Protein gels. Advances in Protein Chem. **4**: 1-74.
- KASSEL, R. & M. J. KOPAC. 1953. Experimental approaches to the evaluation of fractionation media. I. The action of ions on the protoplasm of *Amoeba proteus* and *Pelomyxa carolinensis*. J. Exptl. Zool. **124**: 279-302.
- KASSEL, R. & M. J. KOPAC. 1954. Experimental approaches to the evaluation of fractionation media. II. The effects of various added substances on amoeba cytoplasm. J. Exptl. Zool. **126**: 497-510.
- KOPAC, M. J. 1943. Micrurgical and Germ-free Techniques. : 26-71. Thomas. Springfield, Ill.
- KOPAC, M. J. 1948. The action of aromatic amidines and nucleates on the interfacial denaturation of proteins. Acta Unio Intern. contra Cancrum. **6**: 357-365.
- KOPAC, M. J. 1950. The surface chemical properties of cytoplasmic proteins. Ann. N. Y. Acad. Sci. **50**(8): 870-909.
- KOPAC, M. J. 1951. Probable ultrastructure involved in cell division. Ann. N. Y. Acad. Sci. **51**(8): 1541-1546.
- LAZAROW, A. 1945. The chemical structure of cytoplasm as investigated in Professor Bensley's laboratory during the past ten years. Biol. Symposia. **10**: 9-26.

METHODS FOR CORRELATED OPTICAL AND ELECTRON MICROSCOPIC STUDIES OF AMOEBAE*

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The study of the architecture of life with the electron microscope is severely hampered by two major instrumental limitations. The first of these is the requirement that all specimens be placed in vacuum, immediately eliminating the possibility of making direct, dynamic studies of living protoplasm. However, even if this limitation were to be overcome by some as yet unforeseen technical development, the second requirement would definitely prevent the examination of most living specimens. This requirement, imposed by the poor penetrating power of the electron beam, dictates that the specimen be about $0.1\ \mu$ or less in thickness, a qualification possessed only by the viruses. As a direct result of these two stringent limitations, the most popular method for studying cells and tissues in the electron microscope is to fix them, embed them in a suitable matrix, and cut them into unimaginably thin sections. Only about a decade ago it was believed by expert microtommists that sections much less than $1\ \mu$ in thickness were almost unattainable, but advances in the art of thin sectioning have been so rapid that now most electron microscope laboratories routinely cut sections $0.025\ \mu$ thick. Such a dimension can be translated into something more tangible by saying that we are now able to cut a single human red blood cell into more than 300 slices. Thus the best the cytologist can hope to see in the electron microscope is a static view, in black and white, of a thin, almost two dimensional slice of what is left of a cell after it has been modified chemically by fixation, dehydration, and embedding, abused mechanically by sectioning, and altered in various ways in the high energy of an electron beam.

Amoebae present several difficulties that are usually not of great importance in studying most other types of cells. Their large size is a definite handicap. It would require 20,000 sections to examine completely a single *Amoeba proteus* just large enough to fit into a sphere $0.5\ \text{mm.}$ in diameter. It would require about 40 man days of work merely to make electron micrographs of this many sections. The continuous, dynamic mobility and constantly changing shape of the amoebae are indicative of their complete lack of symmetry. Indeed, it is frequently a matter of definition as to whether something is "inside" or "outside" of the cells. Lack of symmetry or well-defined shape leads to unusual difficulties in orientation when examining single sections in the electron microscope. Again, the appearance in thin sections of inclusions that are normally

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found in amoebae may be extremely deceptive, in the sense that sometimes it is difficult to distinguish between amoebal substance and the wide variety of materials that are normally ingested by the organism. These ingested materials include hard, crystalline objects, possibly bits of fine sand, that ruin the cutting edges of the glass knives normally used and form a tremendous handicap to proper thin sectioning.

In view of these difficulties, it would be desirable to have methods and instruments at our command that would fulfill the following requirements: (1) permit the selection of a particular portion of a single amoeba for study in the electron microscope after having studied it in both the living and embedded conditions with an optical microscope; (2) preserve, as precisely as possible, all of the structures visible in living amoebae through the entire embedding process; and (3) permit the cutting and mounting of ribbons of serial sections, approximately $0.025\ \mu$ in thickness, regardless of how hard the inclusions in the amoebae may be. The methods presented here were designed to meet the above requirements.

In preliminary experiments on methods for fixation, it was found that when suspensions of amoebae were poured into a 1 per cent osmium tetroxide solution in distilled water, many of them developed peculiar surface blebs. Moreover, when handled in suspension, the organisms suffered a great deal of breakage during the dehydration and embedding procedures because of the brittleness of the fixed protoplasm. It was also difficult to check their condition with an optical microscope during the dehydration and impregnation phases of the embedding process. By taking advantage of some of the natural tendencies of the organisms, we were able to induce them to fasten themselves to the surface of a microscope slide where they could be handled with no danger of breakage through the entire fixing and embedding procedure. This was done simply by starving them for about 24 hours, after which several dozen were placed on a microscope slide and fed with a culture of *Paramecium aurelia*. While feeding, many of the amoebae naturally fastened themselves to the slide. Most of the fluid covering the amoebae was then poured off and, almost simultaneously, a quantity of a 1 per cent solution of osmium tetroxide in distilled water was poured over the slide. It was found that it was extremely important to immerse the amoebae in the full strength of the fixative in the shortest possible time. Even the slightest diffusion gradient, lasting only a few seconds, was enough to cause a blebbing reaction at the surface of the cell. When the amoebae were suddenly and completely covered by the fixative, no change in their appearance could be detected with a stereo-binocular dissecting microscope at magnifications up to 80 times or with compound microscopes at higher magnification. Paramecia that had been freshly trapped in large vacuoles in the amoebae became immobilized almost immediately, indicating that the fixative penetrated through the amoebal protoplasm with great rapidity. We believe that this is the first time that a living system has been used as an internal indicator of the rate of penetration of a fixative through a cell or tissue.

The amoebae were fixed for a minimum time of 3 min., after which the fixative was washed off with a tap water rinse, and the slides were placed in Coplin jars

containing 50 per cent acetone. Dehydration was effected with a series of increasing concentrations of acetone (50, 70, and 100 per cent) with 3 final changes in 100 per cent acetone, allowing a minimum of 5 min. in each change. The specimens were then impregnated with a methacrylate monomer mixture containing 35 per cent methyl methacrylate, 45 per cent *n*-butyl methacrylate, 0.50 per cent Luperco CDB, and a trace of Aerosol OT, using 2 changes of about 5 min. each with storage in a third change until ready for embedding.

Embedding was accomplished by a new "open-face" technique.¹ A quantity of the same monomer mixture used for impregnation was partially polymerized to a high viscosity in a drawn-out test tube according to the method described by Borysko and Sapranaukas.² The microscope slide holding the impregnated specimens was placed on a rectangle of aluminum foil, specimen side up, and the foil was folded around the slide to form a shallow tray with walls extending 2 to 3 mm. above the upper surface of the slide. The tray was then filled with partially polymerized methacrylate and placed in a cold (room temperature)

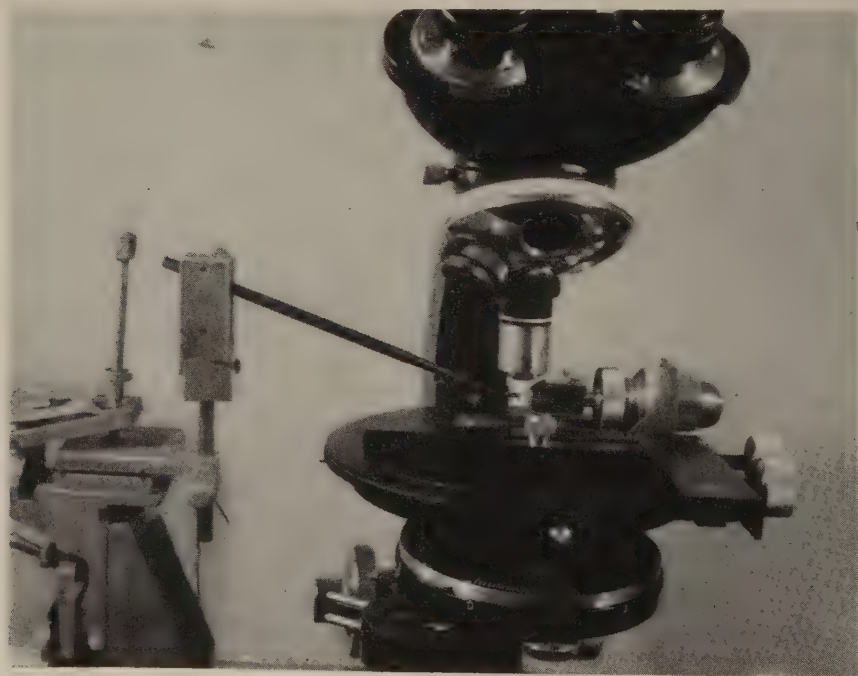


FIGURE 1. Equipment used for trimming embeddings accurately so that a particular small portion of an amoeba can be selected for thin sectioning. The micromanipulator at the left holds a tapered rod with a fragment of razor blade cemented with collodion to its free end. The embedding is held in the specimen clamp of a Porter-Blum Ultramicrotome, mounted on a special fitting attached to the mechanical stage of a Zeiss-Winkel rotating stage microscope. The trimming assembly is designed so that it can be mounted on or removed from the microscope in a matter of seconds. The motion of the razor blade fragment is restricted to a single direction. Cuts at different angles through the embeddings are made by rotating the stage of the microscope.



FIGURE 2. Close-up of the trimming assembly showing details of the relationship of the flat embedding to the vertically held fragment of a razor blade. Using a $\times 10$ objective lens, it is possible to trim an embedding to within a few microns of any desired part of the cell in less than 15 min.

oven. The oven was turned on and the temperature was allowed to rise freely to a peak of 95° to 105° C. This normally was achieved in about 2 hours. The embeddings were "soaked" at this high temperature overnight to drive the polymerization reaction to completion, after which the oven was turned off and allowed to cool slowly to room temperature before it was opened. At this stage in the process, the specimens could be examined carefully with an optical microscope by simply stripping off the aluminum foil. In the perfectly transparent embedding matrix, any gross damage to the specimen could be detected easily.

The embeddings were prepared for sectioning by first lifting them off the microscope slide, using a razor blade to pry the acrylic away from the glass in the form of a transparent slab. A single amoeba was then selected for sectioning by cutting a small rectangular piece out of the slab with the desired cell at one end. The rectangle was clamped in the specimen holder of the thin sectioning microtome* and excess acrylic was trimmed (freehand) away from the amoeba with a razor blade. Final precise trimming was done by the method described by Borysko.³ The specimen was mounted on a rotating stage microscope and the embedded amoeba was trimmed with a fragment of razor blade held in a micromanipulator (FIGURES 1 and 2), so that whatever structure was

* Porter-Blum microtome,⁴ manufactured by Ivan Sorvall, Inc., Norwalk, Conn., was used exclusively.

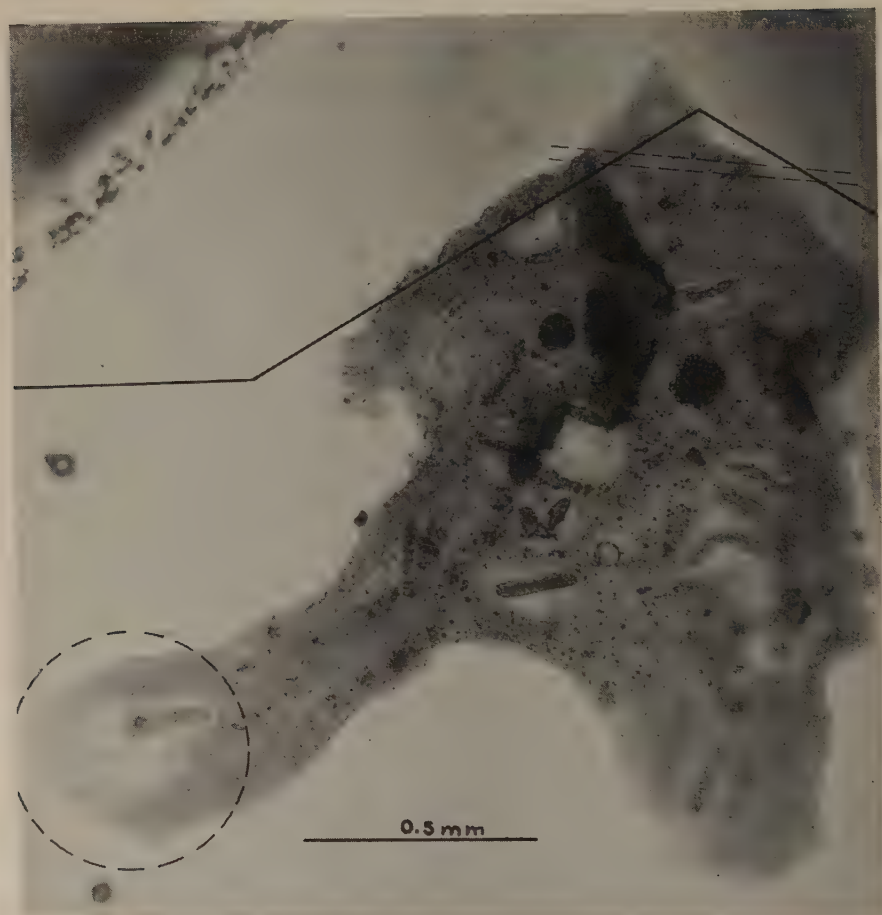


FIGURE 3. Master photomicrograph of an embedded *Chaos chaos* containing fourteen freshly ingested ciliates (*Paramecium aurelia*). The embedding was trimmed to a pyramid as shown by the solid line, and thin sections containing cross sections of a ciliate (out of focus in picture) were cut in the region between the two broken lines. The paramecium in the long pseudopod in the lower left quadrant (broken circle) is shown in FIGURE 6 as it appeared in a polarization microscope. $\times 119$.

desired appeared in the apex of a small pyramid.* A master photomicrograph was made of the whole amoeba prior to trimming, and the outline of the trimmed pyramid was drawn on a print of this picture. After a number of thin sections were cut and mounted, the embedding was examined optically; and the region of the pyramid included in the sections, as well as the precise plane of sectioning, was determined and noted on the master picture (FIGURE 3).

* The micromanipulator was custom built for G. O. Gey, The Johns Hopkins Hospital, Baltimore, Md., who kindly loaned it to E. Borysko. It is not available commercially.

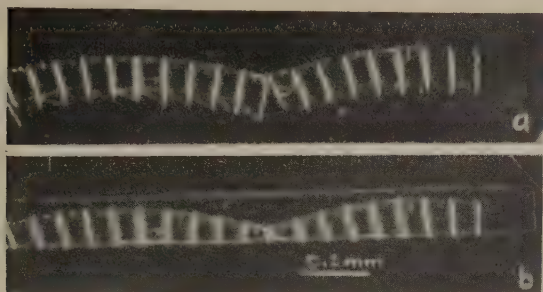


FIGURE 4. (a) Ribbon of sections as seen with dark field illumination during the serial section mounting process. The specimen lies close to the straight edge of the ribbon. The sections are so thin that they are virtually invisible by transmitted light. (b) The same ribbon after mounting so that the straight edge lies in the slot of the grid. The width of the slot is 0.07 mm.

The pyramids were intentionally trimmed so that, when ribbons of sections were cut, one edge of the ribbon was straight while the other was saw-toothed (FIGURE 4). The straight edge represents the surface of the embedding that was in contact with the microscope slide on which the amoeba were fastened. All of the sections examined in this work were mounted serially on slot-type "grids" with the aid of a micromanipulator* (FIGURE 5). The ribbons consisted of from 2 to 30 or more sections, the number depending entirely on chance. They were always mounted with the straight edge in the slot (FIGURE 4), since the embedded amoebae are known to be closer to this than to the saw-tooth edge. This also gives orientation with respect to gravity, the straight edge representing the "down" side of the section.

The sections were cut with a diamond knife of superb quality, furnished by Humberto Fernandez-Moran, Venezuelan Institute of Neurological and Brain Research, Caracas, Venezuela.⁵ The knife has a near-perfect edge that is apparently impervious to damage by the many hard inclusions in the amoebae and seems to cut both "soft" and "hard" materials with equal facility. In our opinion, the development of the diamond knife is one of the most important contributions to the art of thin sectioning.†

Discussion

The results presented here were obtained from a very brief and by no means complete study of one amoeba (*Chaos chaos*). Other amoebae were studied, of course, but it was felt that the effectiveness of the techniques could be demonstrated best by confining the discussion to the information derived from thin sections of a very small portion of a single cell. It is not our intention to present a detailed cytological study here: we propose only to bring up a few points that emphasize the value of the methods used.

The amoeba in question, shown in the embedded state in FIGURE 3, had been fed with a culture of *P. aurelia* for about 15 min. prior to fixation. In this short

* The slot-type grids were made to order by Smethurst High-Light Ltd., Bolton, Lancaster, England. They are now available as a stock item.

† Diamond knives are not available commercially at this time.



FIGURE 5. Placing a slot-type grid on a ribbon of sections. The sections had been collected previously and allowed to dry down on a collodion-coated microscope slide. After the collodion film was floated off the slide with water, a slot-type grid, held on the end of a drawn-out glass rod tipped with adhesive, was located over the ribbon of sections, using the same micromanipulator shown in FIGURE 1. The ribbon was oriented in the slot by rotating the microscope stage, when the grid was lowered until it touched the collodion film.

time, the amoeba ingested 14 paramecia, all of which were still alive just prior to fixation, as evidenced by the beating of their cilia. Immediately after immersion in a 1 per cent solution of osmium tetroxide, the amoeba was examined with a microscope for evidence of fixation damage. Although all evidence of life, such as, cytoplasmic streaming and the ciliary beat of the paramecia had disappeared, the organism looked exactly as it did prior to fixation. This examination was repeated with both ordinary and polarized light after embedding, and again no evidence of damage or distortion during preparation could be detected other than the shrinkage that is known to occur during dehydration.³ In the polarizing microscope, the strong birefringence of the pellicle of the paramecia was seen to be well preserved (FIGURE 6). Furthermore, examinations with polarized light revealed the fact that the embedding matrix was free of internal strain, supporting the earlier observation by Borysko⁸ that the use of high polymerization temperatures provided a means for relieving internal stresses caused by polymerization contraction.

Thus, knowing at the start that our specimen was well preserved, we could proceed with confidence to the examination of sections of particular portions of the organism in the electron microscope. The amoeba was trimmed to a small pyramid that included one of the paramecia (FIGURE 3). The pyramid

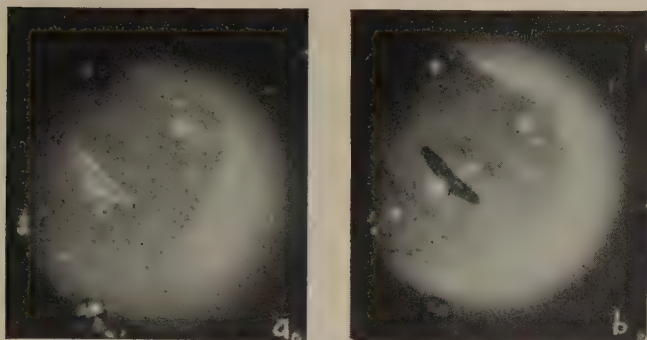


FIGURE 6. Appearance of an embedded *P. aurelia*, shown in lower left quadrant of FIGURE 3, in a polarization microscope, illustrating the preservation of the normal birefringence of the living pellicle.

was intentionally cut so that almost exact cross sections of the paramecium would be included in the sections, as can be seen in the low magnification view of a portion of one of the sections shown in FIGURE 7. A portion of a cross section through the paramecium appears on the right hand side of the picture completely surrounded by the cytoplasm of the amoeba. It is evident that most of the water that had been ingested with the paramecium has already been expelled, although there does not seem to be any specialized structural entity in the amoebal cytoplasm immediately surrounding the food vacuole, such as the numerous small vesicles generally associated with pinocytosis that could account for the removal of the water. However, the "lumen" of the vacuole was seen to be continuous with several long channels that extended radially away from the vacuole for long distances. One of these channels can be seen in FIGURE 7. It is conceivable that these channels extend to the exterior of the cell, providing an escape route for the water ingested with the paramecium.

Additional details of the fine structure of both the amoeba and the paramecium can be seen at higher magnification in FIGURE 8. Here, the striking difference between the thick, dense, double-membraned pellicle of the paramecium and the simple, delicate single membrane forming the surface of the amoeba in the food vacuole may be seen. In *C. chaos*, there does not seem to be any difference in structure between the membranes surrounding food vacuoles, small or large cytoplasmic vesicles, or even the free cell surface. They all appear as simple, single membranes.

The mitochondria of *C. chaos* characteristically appeared as round or oval bodies approximately $1\ \mu$ in minor dimension, with a complex internal structure consisting of a system of pleated membranes, areas of amorphous material, and numerous small, dense granules (FIGURE 9). From their appearance in a single section one could easily be led to assume that they were predominantly spherical or ovoid in 3-dimensional shape. However, when seen in serial sections (FIGURE 10), it becomes clear that many of them are actually serpentine filaments. With longer ribbons of sections, it may be possible to reconstruct a mitochondrion completely in 3 dimensions.

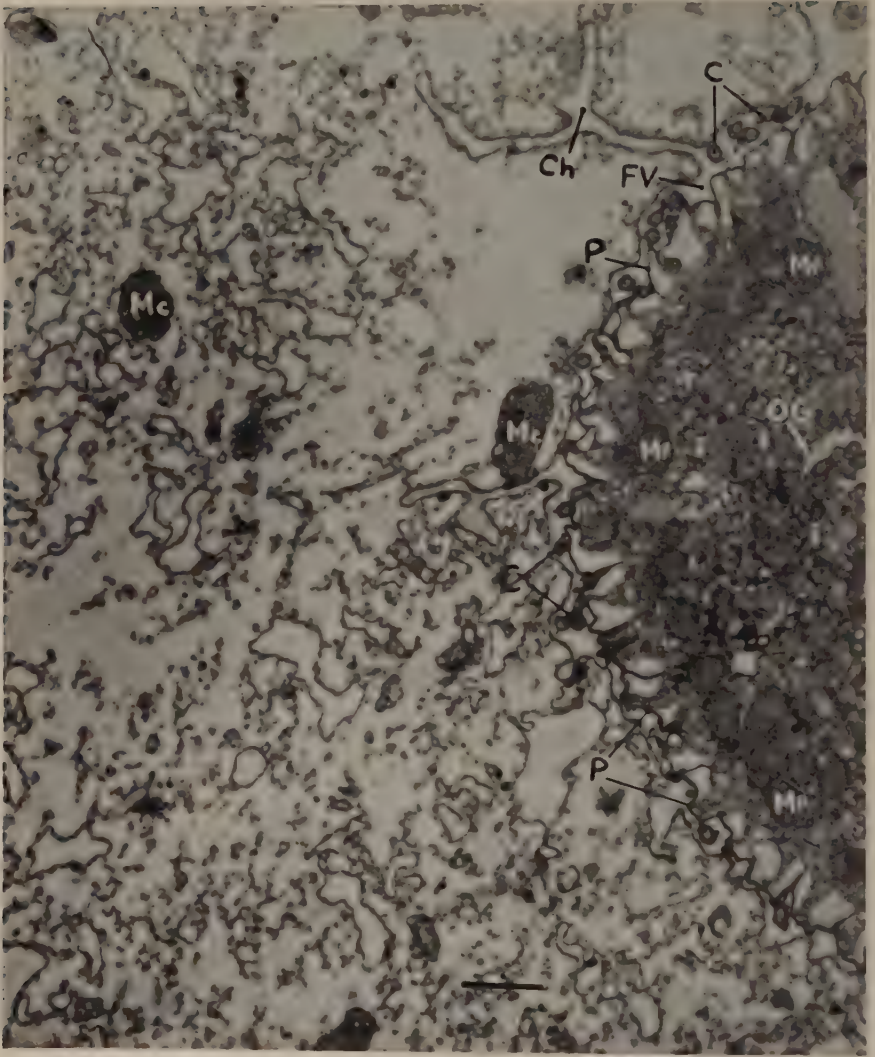


FIGURE 7. Low magnification electron micrograph of a thin section of *Chaos chaos* taken from the region indicated in the master photomicrograph (FIGURE 3). This section includes a portion of a paramecium (in cross section) in a food vacuole, shown on the right side of the picture. Most of the water ingested with the paramecium has been expelled, possibly through channels (*Ch*) that may extend to the exterior of the cell from the lumen of the food vacuole (*FV*). Symbols: *Mc*, mitochondria of the amoeba; *Mp*, mitochondria of the paramecium; *C*, cilia; *P*, pellicle of the paramecium; *OG*, oral groove with cross sections of cilia; *FV*, lumen of the food vacuole. $\times 10,200$.

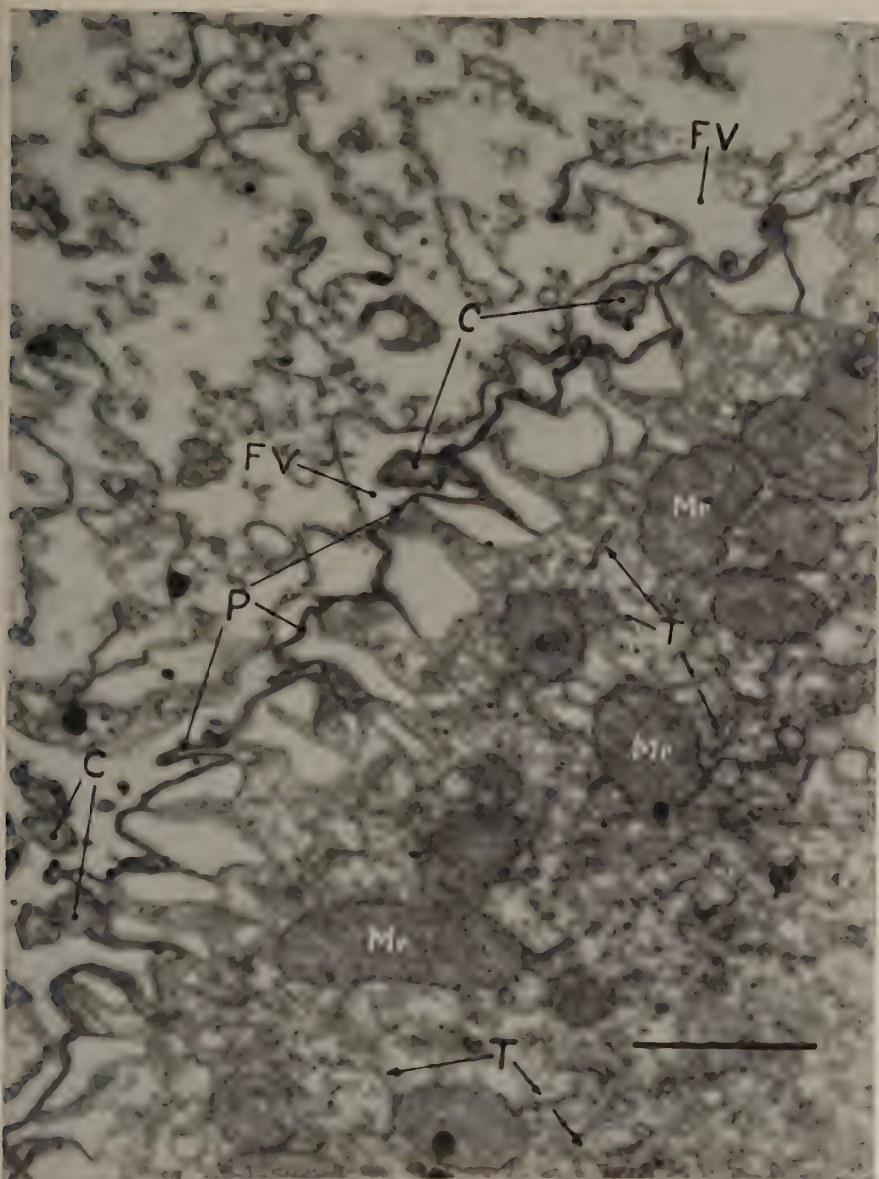


FIGURE 8. Higher magnification view of the edge of the food vacuole containing the paramecium (FIGURE 7). The pellicle (P) of the paramecium extends from the lower left corner to the upper right side of the picture, dividing the area shown in half. The lower right half consists of paramacronal substance, while the upper left half is entirely amoeboid. Here, as well as in FIGURE 7, the relatively "empty" alveolar structure of the amoeboid protoplasm contrasts sharply with the dense, finely granular appearance of the protoplasm of the paramecium. It is interesting to note that the small tubules (T) in the cytoplasm of the paramecium are about the same size as the tubules packed in the mitochondria. $\times 24,000$.

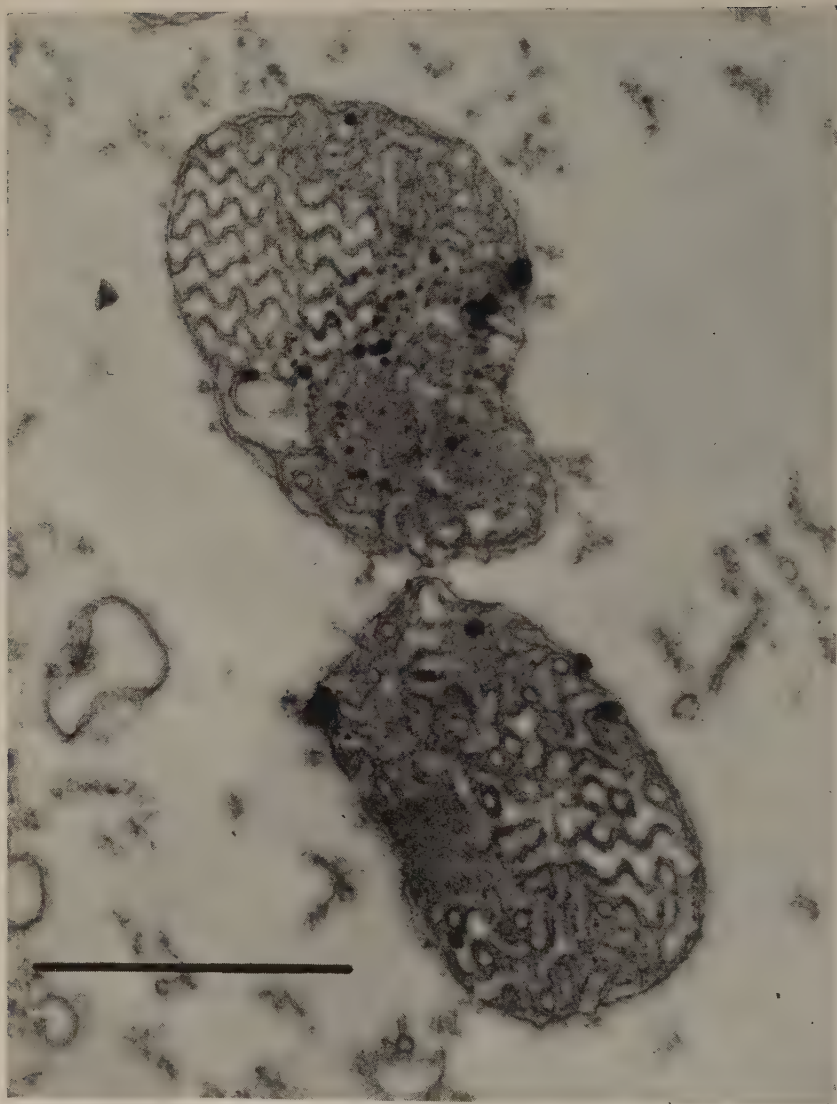


FIGURE 9. Thin sections of mitochondria of *Chaos chaos* showing the highly complex internal structure consisting of parallel wavy membranes, large amorphous areas, small vesicles, and numerous dense granules. $\times 42,000$.

All of the sections examined exhibited a wide variety of dense bodies, some of them obviously crystalline (angular) in shape, others roughly circular. These granules varied in size from less than fifty to several thousand angstrom units. Some of the crystalline bodies proved to be bits of larger crystals contained in a food vacuole in the paramecium that had been shattered by the

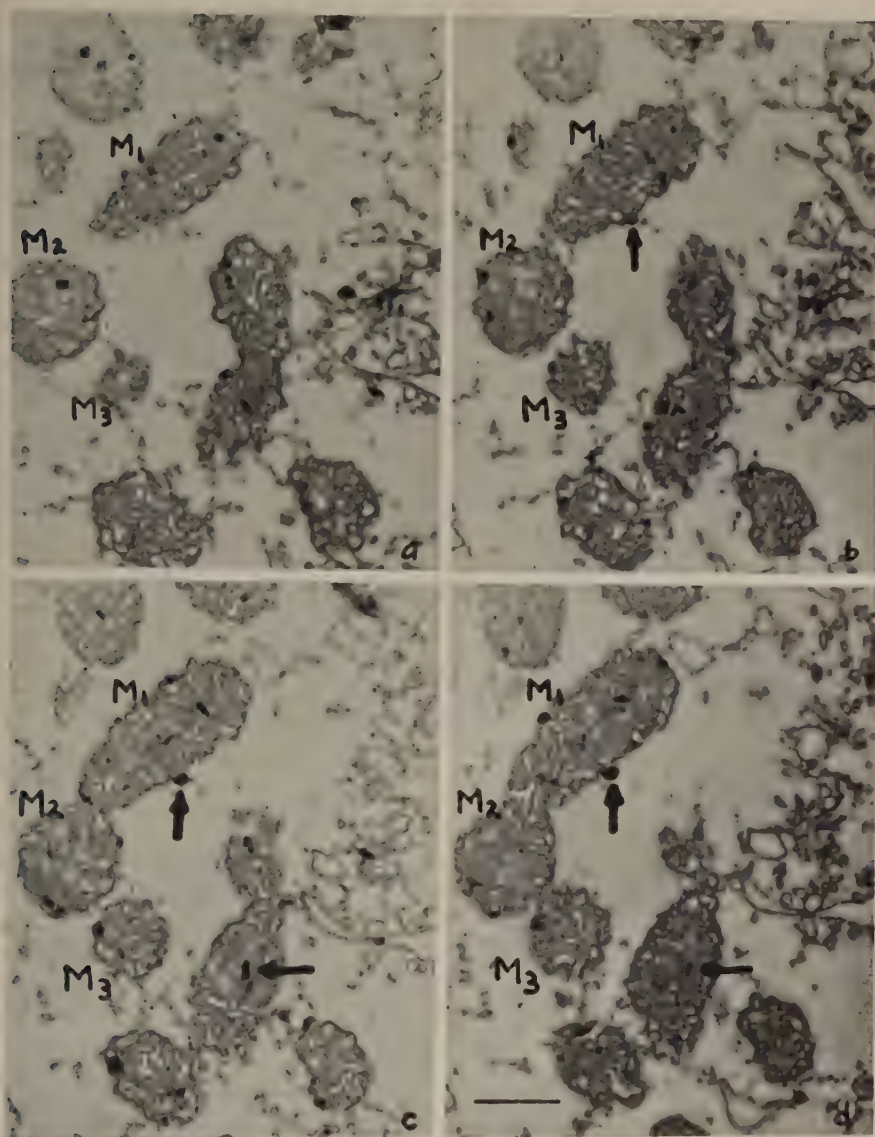


FIGURE 10. Serial sections of a group of mitochondria in *Chaetochaeta*. The three round or oval shaped sections of mitochondria labelled M_1 , M_2 and M_3 in the first section (a) prove to be parts of a single long, serpentine mitochondrion when traced through three additional sections. The dense body on the side of M_1 (vertical arrow) can be traced through three sections, indicating that it was in the embedding prior to sectioning. Similarly, an elongate dense body within a mitochondrion (horizontal arrow) can be traced through two sections. $\times 11,000$.

cutting edge and torn out of the sections, but the other dense bodies could not be distinguished, in single sections, from dirt that normally contaminates a section to a greater or lesser degree. An examination of serial sections was undertaken to establish whether particular dense bodies were actually in or on the sections. In FIGURE 10 several of these dense bodies can be traced through at least three sections, proving that they were in the embedding prior to sectioning. This method is excellent for particles that are several times the thickness of the sections in size, but smaller particles can be identified as part of the sectioned material only when they consistently appear associated with some larger structure. In serial sections, this can be readily determined. FIGURE 11 shows a series of sections through several nucleoli. In each section, clusters of small granules appear in the nucleoli. Although these granules are too small to be traced individually from section to section, the fact that they consistently appear in intimate association with the nucleoli indicates that they were originally present in the embedding and are not a postsectioning contaminant. Small particles of similar size were also constantly found in association with the mitochondria and the surface membrane of the organism.

Although it was definitely established that the majority of these particles were present in the embedding prior to sectioning, this does not mean that they were actually present in the living cell. It could well be that they represent material that was precipitated out of solution during one or more of the phases of processing. At the present time, reliable, direct methods for confirming the existence in the living cell of the smaller structures seen in thin sections in the electron microscope have not been developed. With the exception of a few highly specialized types of small structures such as collagen and red cell ghosts that can be isolated in quantity and examined by other means, for example, X-ray diffraction and interferometry, our confidence in the reliability of our methods for the preservation of fine structure is supported chiefly by optical comparisons between the appearance of the living and embedded cells. In our laboratories constant efforts are being made to detect and eliminate the more obvious artifacts of preparation so that we can interpret more accurately the shadowy image of an inert, lifeless replica of a tiny fragment of protoplasm that we see in the electron microscope. It is our contention that a full understanding of living cells is dependent on our knowledge of the limitations of the methods used to study the cells.

Concluding Remarks

The methods we have described are in conformance with the previously outlined requirements for accurate and efficient electron microscopic studies of the fine structure of amoebae. The immobilization of the cell, with subsequent embedding in a flat slab of perfectly transparent polymer, permits careful studies of the preservation of structure, selection of particular cells, or small portions of a single cell for study in the electron microscope and orientation with respect to gravity in an otherwise asymmetrical system. The use of a diamond knife and the Porter-Blum microtome makes possible the production of ribbons of serial sections from which three-dimensional aspects of the struc-

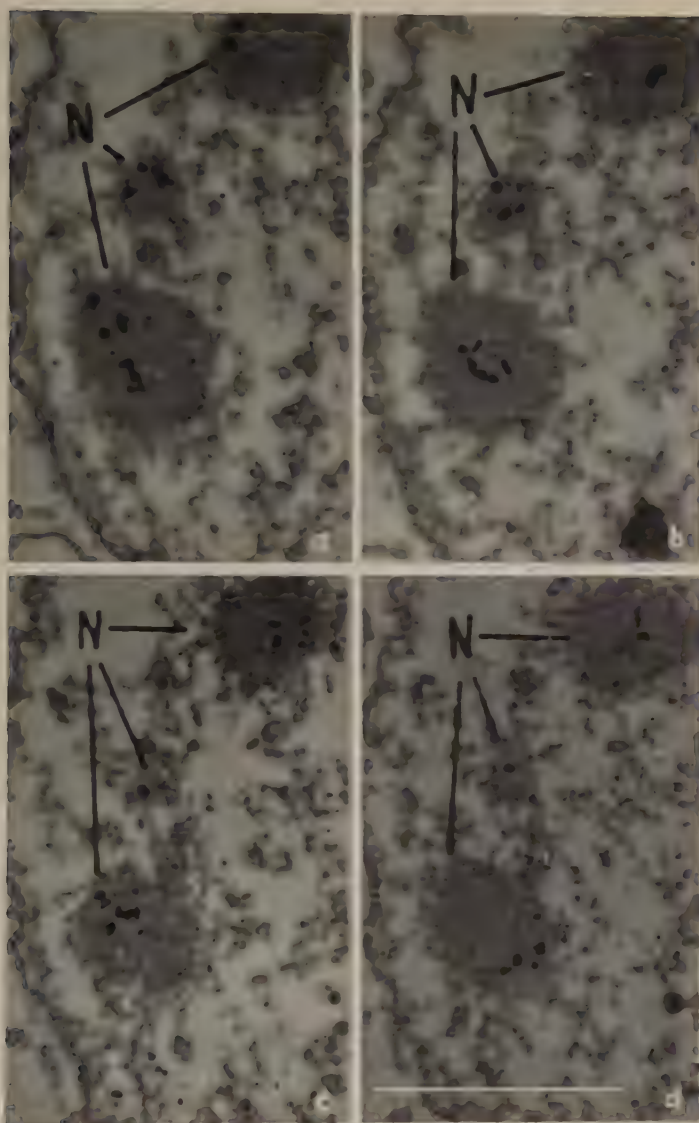


FIGURE 11. Serial sections of a portion of a nucleus of *Ctenocephalides felis* showing several nucleoli (N). A portion of the nuclear membrane appears at the left side of each picture. Each nucleolus contains a cluster of dense granules that are individually too small to be traced from section to section. However, the fact that the granules consistently appear in association with the nucleoli in the serial sections indicates that they are not a postsectioning artifact. $\times 33,000$.



FIGURE 12. Food vacuole (FV) containing bacteria (B) in *P. aurelia* which, itself, lies in a food vacuole in *Clostridium*. Symbols: M, mitochondria; OG, oral groove with cross sections of cilia; T, concentration of cytoplasmic tubules around oral groove. This electron micrograph illustrates the interesting possibility of studying at least three different organisms simultaneously with a single set of thin sections. $\times 24,000$.

ture of small cellular components can be reconstructed. The fourth dimension of time, so very important in dynamic living systems, can readily be added by the proper optical selection of cells or structures in particular phases of activity. Thus, it is now technically possible to study the structure of the amoebal cell in the electron microscope in the same manner that the anatomist studies the gross anatomy of a dog, cat, or cadaver: with full knowledge of the age, physiological condition, experimental history, and manner of death of the specimen and with the ability to select a particular portion of the "body" for detailed "dissection."

An interesting sidelight of these techniques is the fact that the amoebae provide a convenient means for catching and immobilizing free swimming protozoa. The protozoa, in turn, may have caught still smaller organisms, such as the bacteria shown in FIGURE 12. All three organisms may be studied simultaneously in the electron microscope with a single set of thin sections. In view of the high cost of electron microscopic studies, bacteriologists and protozoologists may find collaboration with these methods quite profitable.

References

1. BORYSKO, E. 1959. An "open face" methacrylate embedding technique. In preparation.
2. BORYSKO, E. & P. SAPRANAUSKAS. 1954. A new technique for comparative phase-contrast and electron microscope studies of cells grown in tissue culture, with an evaluation of the technique by means of time-lapse cinemicrographs. *Bull. Johns Hopkins Hosp.* **95**: 68.
3. BORYSKO, E. 1956. Recent developments in methacrylate embedding. I. Study of polymerization damage phenomenon by phase contrast microscopy. II. Methods for sectioning of optically selected single cells, the orientation of the plane of sectioning and the identification of the region of the specimen included in the sections. *J. Biophys. Biochem. Cytol. Suppl.* **2**: 3; 15.
4. PORTER, K. R. & J. BLUM. 1953. A study in microtomy for electron microscopy. *Anat. Record.* **117**: 685.
5. FERNANDEZ-MORAN, H. 1956. Applications of a diamond knife for ultrathin sectioning to the study of the fine structure of biological tissues and metals. *J. Biophys. Biochem. Cytol. Suppl.* **2**: 29.

ELECTRON MICROSCOPE STUDIES ON AMOEBAE

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The free-living amoebae, principally *Amoeba proteus* and *Pelomyxa carolinensis* (*Chaos chaos*) have been widely used in chemical and physiological studies, as this monograph demonstrates. The cytology of both *A. proteus* and *P. carolinensis* has been extensively studied in the light microscope (Andresen, 1956), while electron microscope studies have been confined to *A. proteus*.

The higher resolving power of the electron microscope offers a more precise means of correlating function with structure on the cellular level. Before experimental changes can be evaluated, however, the normal geography of the cell must be thoroughly charted. This can be done properly only by examining many sections from large numbers of amoebae.

Three species of free-living, fresh-water amoebae were studied: *Amoeba proteus*, *Pelomyxa carolinensis* (*Chaos chaos*), and *Hartmannella rysodes*. The fine structure of the plasmalemma, mitochondria, Golgi complex, endoplasmic reticulum, contractile vacuole, and other organelles are described, as well as the cytoplasmic matrix, the nuclear envelope, and contents of the nucleus.

Materials and Methods

Amoeba proteus and *Pelomyxa carolinensis** and *Hartmannella rysodes*† were washed several times in spring water and then packed into a pellet by centrifugation. They were fixed for a period of 5 to 10 min. in 1 per cent OsO_4 in veronal acetate buffer, pH 8.6, with 0.01 per cent CaCl_2 added. A 1 per cent OsO_4 buffer, having a molarity of 1/1400 and a final pH of approximately 8.6, was also used occasionally. Fixation was carried out at 4° C. In some instances, single cells of *P. carolinensis* were fixed and carried through the entire procedure individually.

The amoebae, still packed in a pellet, were dehydrated in alcohol and embedded in *n*-butyl methacrylate. Thin sections were cut on a Porter-Blum microtome, and collected on Formvar-coated specimen grids. An RCA-EMU-3C electron microscope was used for examining the sections.

Observations

Plasmalemma. The limiting membrane or plasmalemma of *A. proteus* has fine fibrous extensions on its outer surface. The electron micrographs in FIGURES 1 and 2 show sections cut through the plasmalemma: at a right angle at A and obliquely at B. The thickness of the plasmalemma, measured when it is cut at a right angle, is approximately 200 Å. The outer filaments extend 1100 to 1700 Å, while the diameter of the filaments is about 80 Å.

* Obtained from the Carolina Biological Supply Co., Elon College, N. C.

† Supplied by S. H. Hutner, Haskins Laboratories, New York, N. Y.



FIGURE 1. Electron micrograph of a section of the plasmalemma of *Amoeba proteus*. The outer portion of the plasmalemma has fine fibrinous extensions. The section is cut normal to the plasmalemma at A, while at B it is cut tangentially. $\times 21,000$.

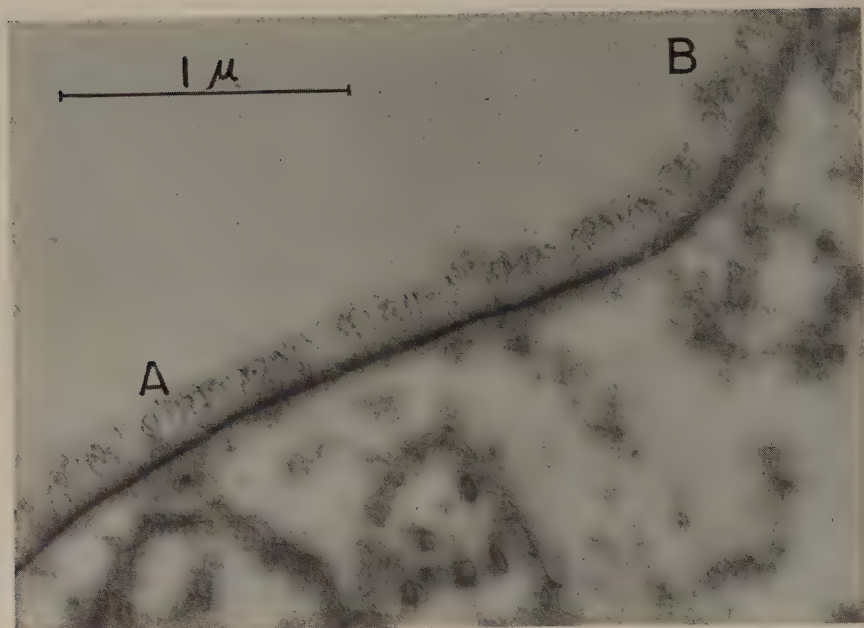


FIGURE 2. Electron micrograph of a higher magnification of the plasmalemma of *Amoeba proteus*. A cross section of the pellicle is seen at *A*, while a tangential view is seen at *B*. The thickness of the plasmalemma at *A* is about 200 Å. The outer filaments extend about 1100 Å. The diameter of the filaments is about 80 Å. $\times 38,500$.

The plasmalemma of *P. carolinensis* is essentially similar to that of *A. proteus*. The diameter of the filaments however, is less, measuring approximately 40 to 60 Å (FIGURE 3).

H. rysodes does not possess a plasmalemma; instead, it is invested with a single, thin, cell or plasma membrane, about 60 Å thick (FIGURE 4).

Endoplasmic reticulum. The cytoplasm of *A. proteus* and *P. carolinensis* is filled with alveolar structures. The great majority of these rounded and elongated saclike vesicles (FIGURE 5) are smooth-surfaced and do not contain granules of the endoplasmic reticulum. Occasionally, however, in some tangential views that show a large surface area, a few granules are seen arranged in whorls (FIGURE 6).

In *H. rysodes*, the endoplasmic reticulum resembles that described in vertebrate cells (Palade and Porter, 1954) and in protozoa (Wolken and Palade 1953; Rudzinska and Porter, 1954; Sedar and Porter, 1955); that is, it appears as a system of flattened vesicles or cisternae. The granular component of the endoplasmic reticulum (Palade, 1955) may be found on the membranes of some of these vesicles (FIGURES 4, 7, and 8). In addition, unattached granules may be seen in the cytoplasm.

Golgi complex. Some areas of the cytoplasm of the three species of amoebae contain an array of parallel membranes (FIGURES 6 and 9). These membranes

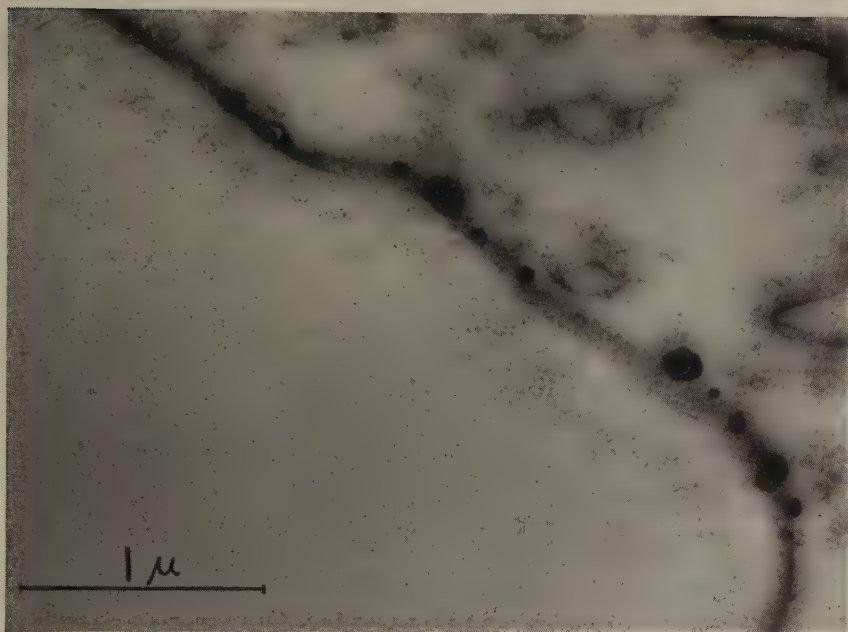


FIGURE 3. Electron micrograph of the plasmalemma of *Pelomyxa carolinensis*. The plasmalemma is about 200 Å thick. The filaments extend about 1700 Å. The diameter of the filaments is about 40 to 60 Å. $\times 32,000$.

are smooth with no particles or granules adhering to their surfaces. The membranes, about 60 Å thick, make up the walls of flattened vesicles that are packed very closely together. These areas have been identified in metazoan cells as the submicroscopic structure of the Golgi complex. A single Golgi complex unit in *P. carolinensis* is small, measuring about 0.5 to 1 μ in area (FIGURES 6, 9, and 10).

Mitochondria. The structure of the mitochondria of *A. proteus* appears similar to that described in other protozoa (Sedar and Rudzinska, 1956; Powers *et al.*, 1956). The mitochondria are made up of an outer limiting membrane and an inner membrane that has fingerlike extensions or villi projecting irregularly into the stroma of the mitochondrion (FIGURE 5). Some of the mitochondria of *P. carolinensis* are similar to those of *A. proteus* in possessing villi formed by fingerlike projections of the inner mitochondrial membrane (FIGURES 11 and 12). However, other mitochondria found in *P. carolinensis* have a more complicated organization, producing strikingly complex patterns (FIGURES 11 and 13), formed by the inner mitochondrial membrane (FIGURE 13).

In addition to these patterns, fine filaments about 40 Å wide are seen in the stroma of some mitochondria (FIGURE 12). These filaments are apparently unrelated to the villi and folds of the inner mitochondrial membrane.

The mitochondria of *H. rysodes* are similar to those of *P. carolinensis*. In FIGURE 4, the mitochondria are seen in close association with lipid bodies found

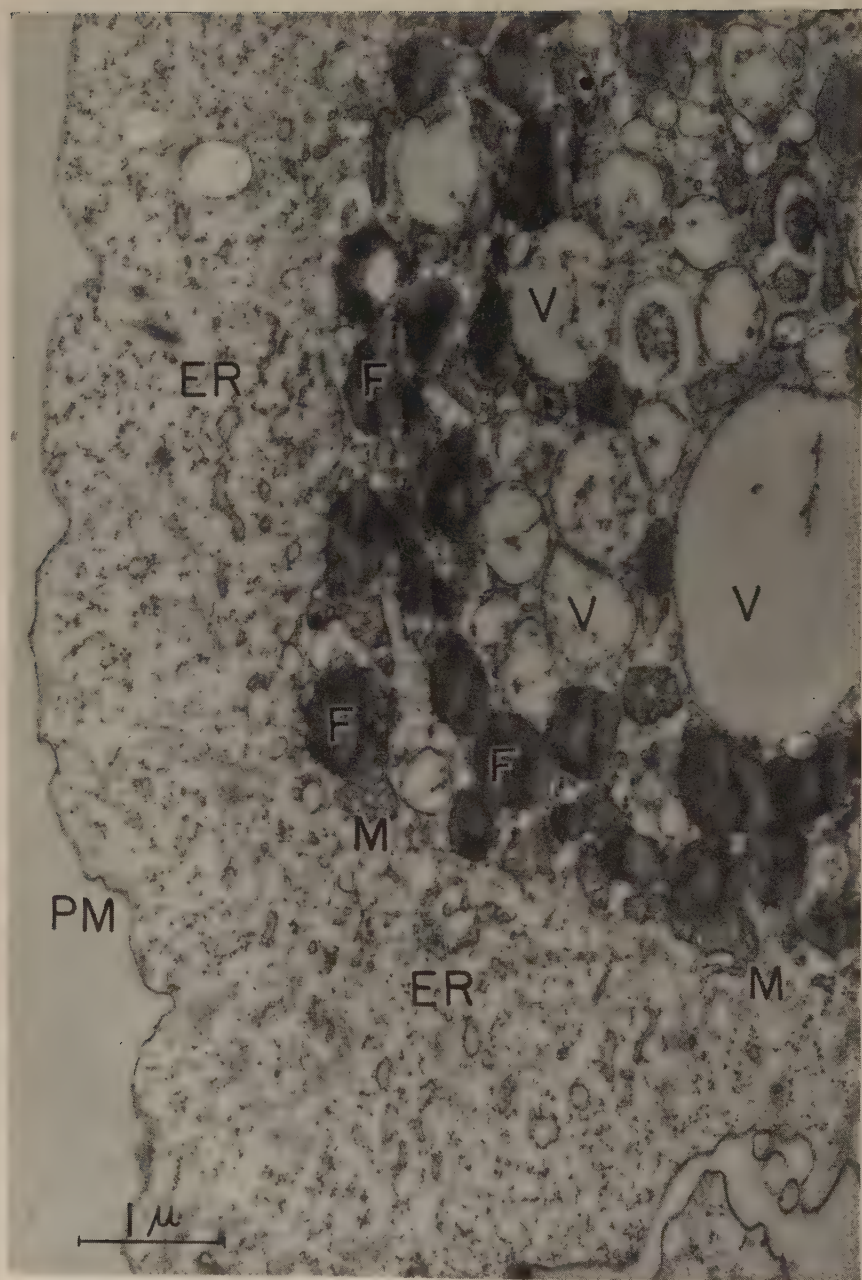


FIGURE 4. Section showing a portion of the cytoplasm of *Hartmannella rysodes*. The plasma membrane (PM) is about 60 Å thick. Elements of the endoplasmic reticulum (ER) are found scattered throughout the cytoplasm. Many lipid bodies (F), vacuoles (V), and mitochondria (M) are found. The mitochondria have a very dense stroma and appear in close association with the lipid bodies. $\times 19,500$.

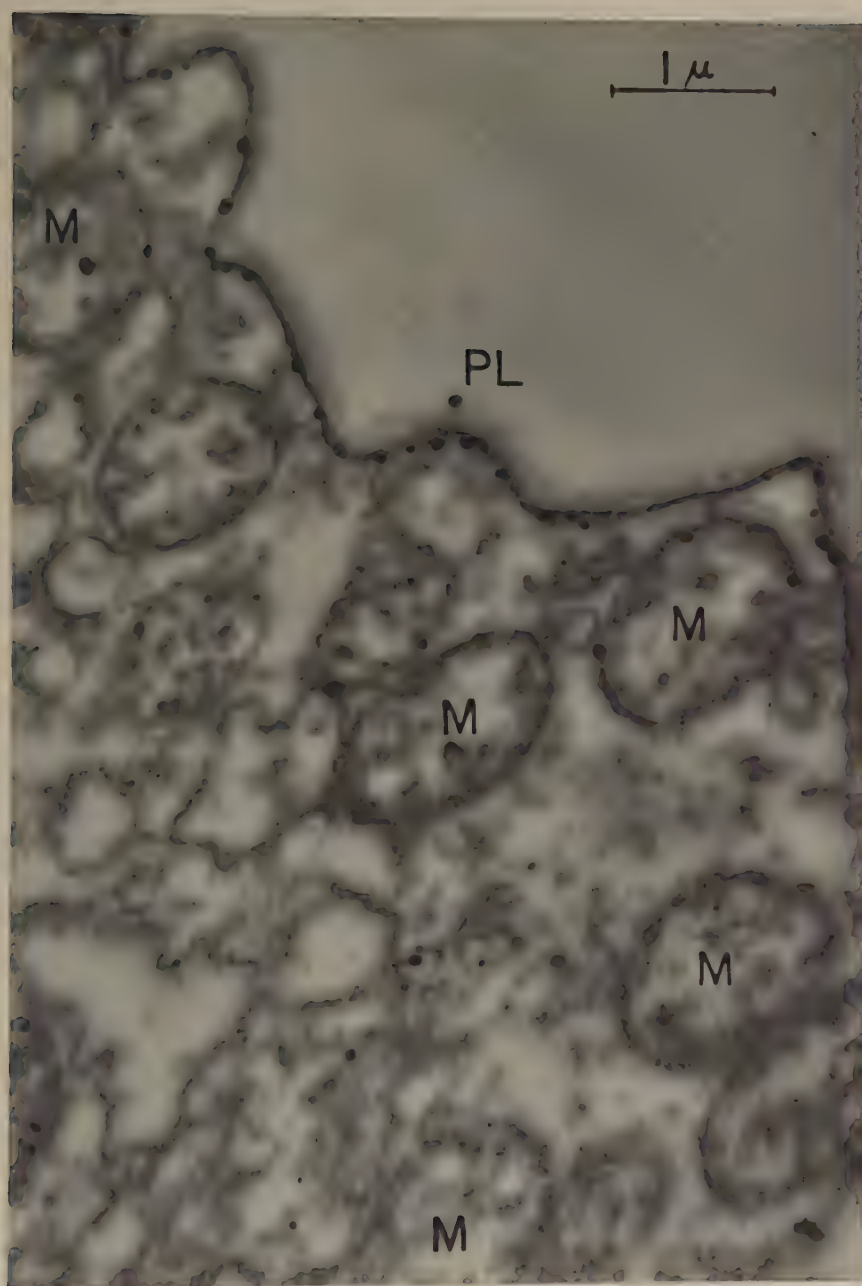


FIGURE 5. Electron micrograph of a section of *Amoeba proteus*. The plasmalemma (PL) is seen with its fibrous outer layer. Mitochondria (M) are scattered throughout the cytoplasm. The cytoplasm is filled with alveolar structures of varying shapes and sizes. $\times 21,000$.

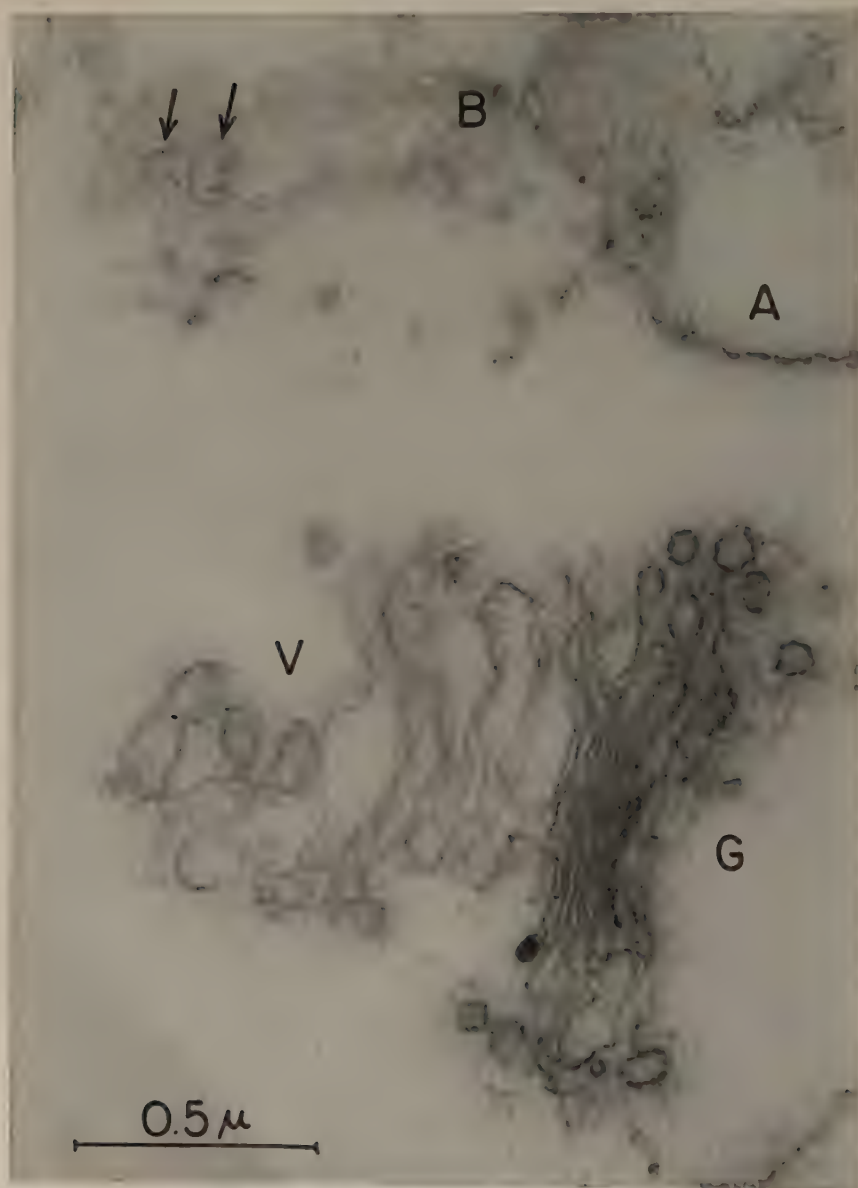


FIGURE 6. High magnification of a Golgi complex (G) of *Pedomyza carolinensis*. The walls of tightly packed flattened vesicles appear as a series of parallel membranes (G) about 60 Å thick. An area of less densely packed vesicles is found at V. A tangential section showing a large surface area of an elongate alveolus is seen at B. At the arrows, particles adhering to the surface can be seen arranged in whorls. The membrane of the alveolus is shown in cross section at A. $\times 64,500$.

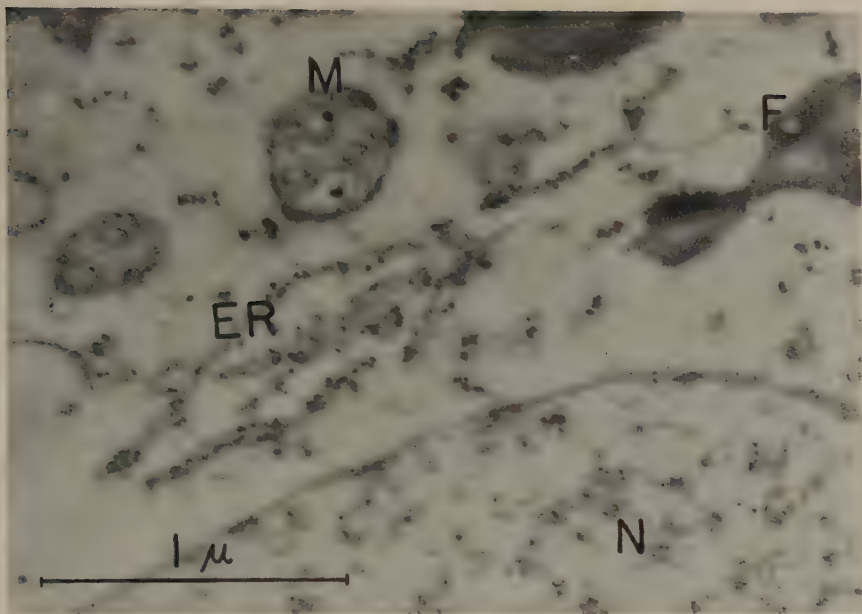


FIGURE 7. Electron micrograph of a portion of the cytoplasm of *Hartmannella rysodes*. Profiles of the endoplasmic reticulum (ER) with granules adhering to the surface of the flattened vesicles or cisternae can be seen. Similar granules are found free in the cytoplasm. A portion of the nucleus (N), as well as mitochondria (M), and lipid bodies (F) can be seen. $\times 40,000$.

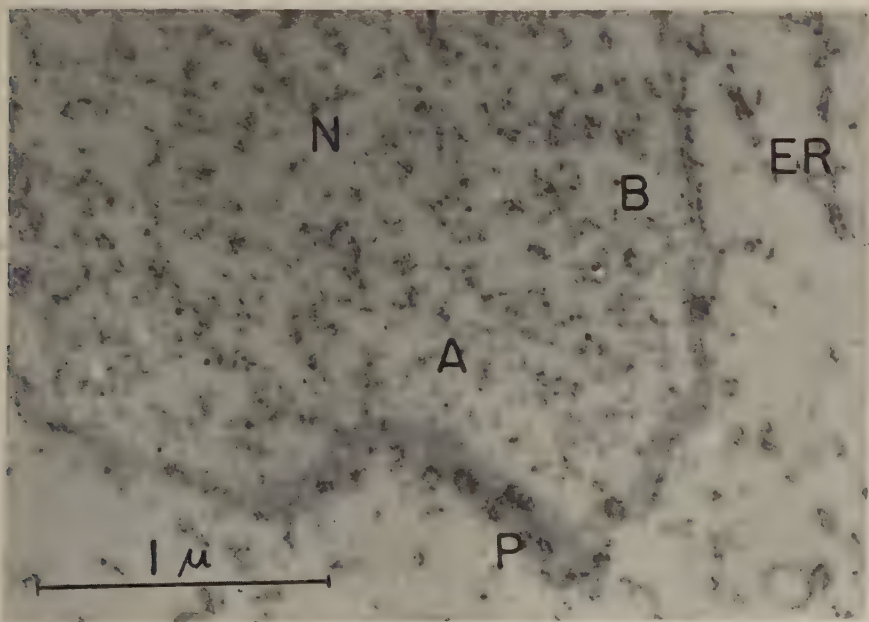


FIGURE 8. Electron micrograph of a portion of the nucleus (N) of *Hartmannella rysodes*. The section is cut normal to the nuclear envelope at B, and the two nuclear membranes can be seen. Granules of the endoplasmic reticulum (ER) are found on the outer nuclear membrane. At A, a tangential view of the nuclear envelope is seen. Pores (P) are found in this area. The pores are about 600 Å in diameter. $\times 38,000$.

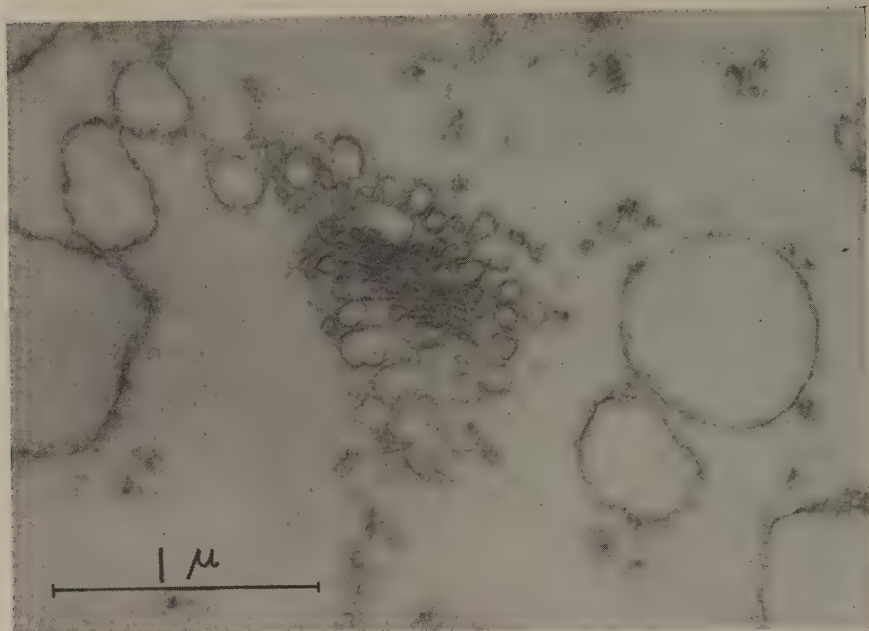


FIGURE 9. Electron micrograph showing an area of closely packed flattened vesicles in *Pelomyxa carolinensis*. Similar areas have been identified in metazoan cells as the submicroscopic structure of the Golgi complex. $\times 35,600$.

in the cytoplasm. The inner area or stroma of the mitochondria of *H. rysodes* is extremely dense (FIGURE 4).

Contractile vacuole. In FIGURE 14 a section cut through a portion of the contractile vacuole is shown. The vacuolar membrane is surrounded by a densely packed layer of small round vesicles. The vesicles in turn are surrounded by mitochondria.

At a higher magnification (FIGURE 10), the vesicles can be seen to range in size from 20 to 200 m μ . The thickness of the contractile vacuole membrane is about 70 Å. At the surface of the contractile vacuole membrane, a vesicle membrane that is continuous with the contractile vacuole wall can be seen (at arrow). The vesicles appear to be emptying their contents into the contractile vacuole; at the same time, their membranes appear to be in the process of becoming incorporated into the contractile vacuole wall.

Food vacuoles. A thick layer of granular material is found in close association with the limiting membrane of newly formed food vacuoles (FIGURE 15). In *Pelomyxa* and *Amoeba*, this granular material appears to be characteristic of a newly formed vacuole. In contrast, the older food vacuoles contain extremely dense, debris-like material, which is found away from the limiting membrane of the highly hydrated vacuole (FIGURE 16).

Crystals and crystal vacuoles. The crystals of amoebae cannot be seen directly in the electron microscope. However, vacuoles that had contained crystals

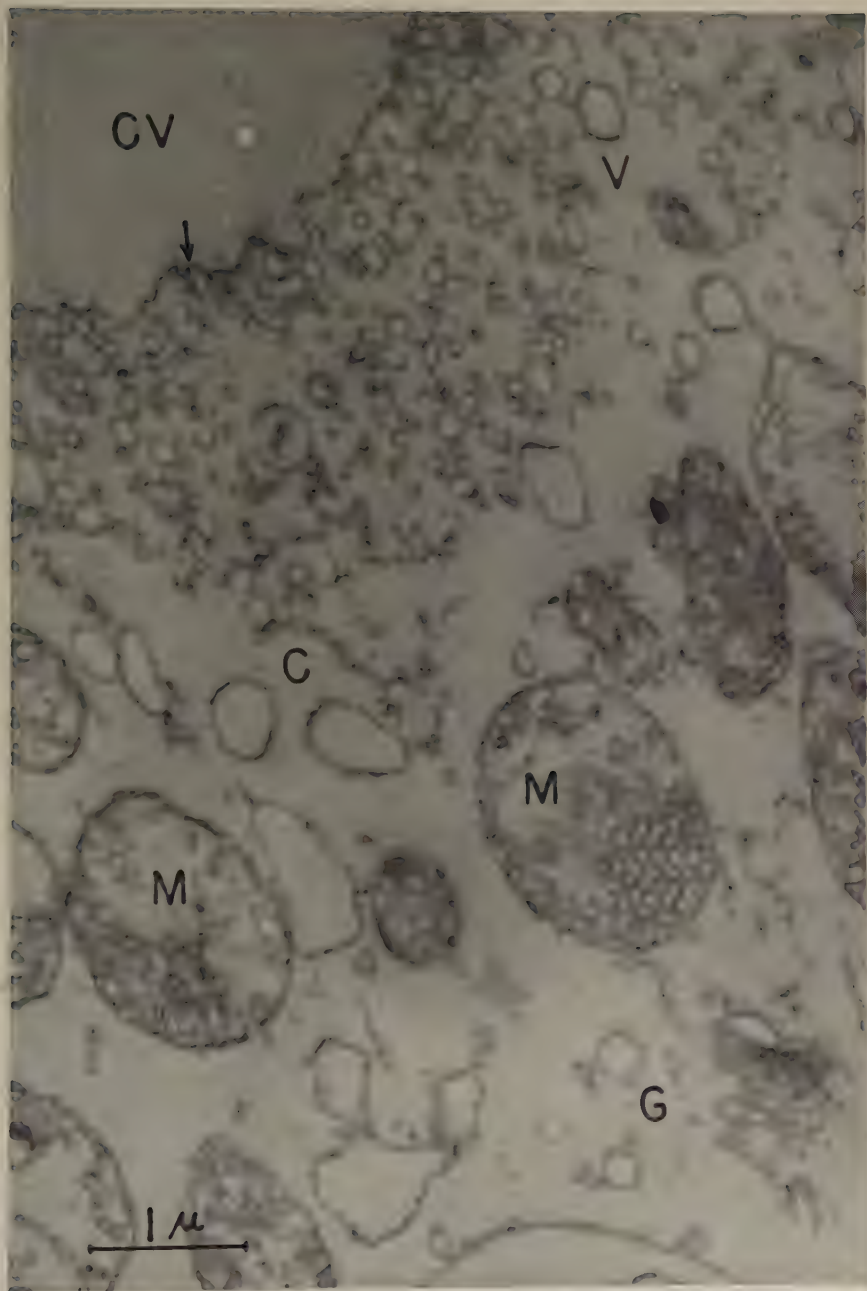


FIGURE 10. Higher magnification of an area of the contractile vacuole shown in FIGURE 14. The vesicles (V) surrounding the contractile vacuole range from 20 to 200 μ m in diameter. The membrane of the vesicle (at arrow) and the membrane of the contractile vacuole are continuous. It is suggested that the vesicles contribute both their contents and their membranes to the contractile vacuole during diastole. A linear arrangement of vesicles is seen at C, indicating a break down of a single larger structure. A single Golgi complex (G) is present at the lower right. $\times 20,250$.



FIGURE 11. Electron micrograph of a group of mitochondria in *Pelomyxa carolinensis*. Sections through mitochondria A, B, and C show different profiles of the complex patterns formed by the inner mitochondrial membrane. Most of the mitochondria show an area of the stroma (S) where there are no projections of the inner mitochondrial membrane. X27,400.

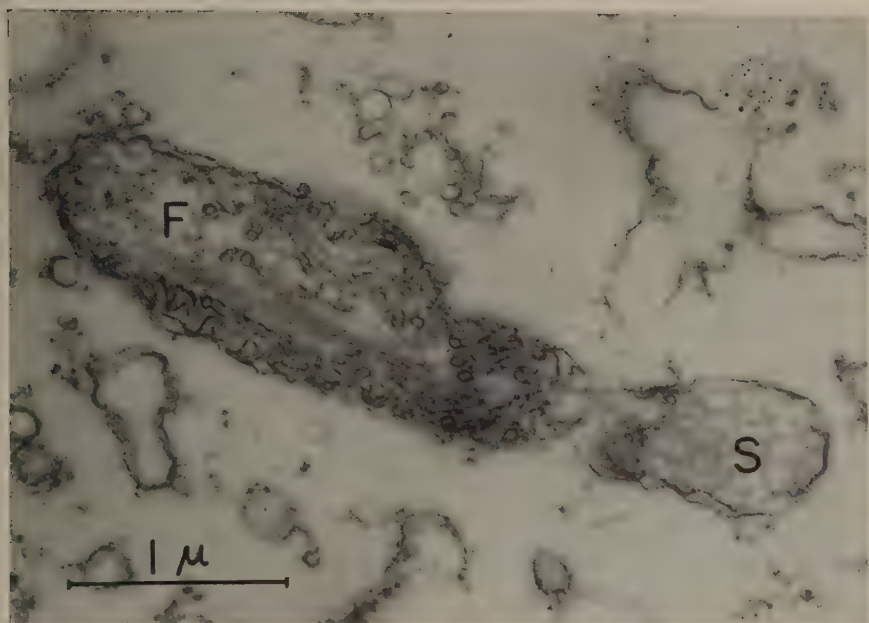


FIGURE 12. Section cut through a mitochondrion of *Pelomyxa carolinensis*. The infoldings of the inner mitochondrial membrane are in the form of villi or fingerlike projections into the stroma. In the stroma, a longitudinal array of fine fibrils (*F*), about 40 Å thick can be seen. The granular appearance of the stromal contents at *S* may represent a cross section through such fibrils. $\times 28,200$.

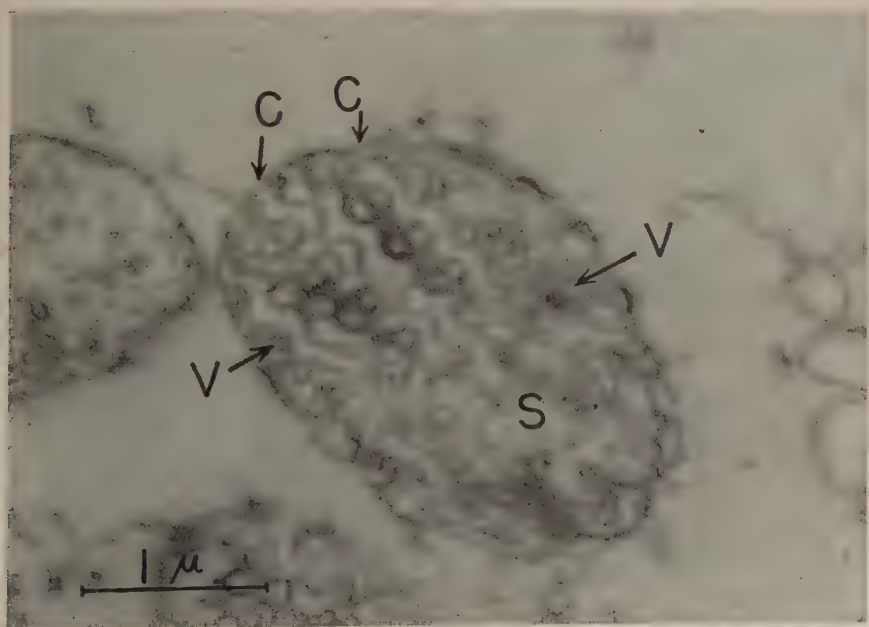


FIGURE 13. This micrograph shows projections of the inner mitochondrial membrane which appear in two forms. Fingerlike projections or villi (*V*) may be seen in cross section. Cristae, or thick shelllike projections of the inner membrane may also be seen (*C*). The regular sequence of the villi (shown in cross section) causes the surface of the cristae to appear wavy. Some villi may be seen projecting into the stroma (*S*) where no cristae are seen. $\times 23,850$.

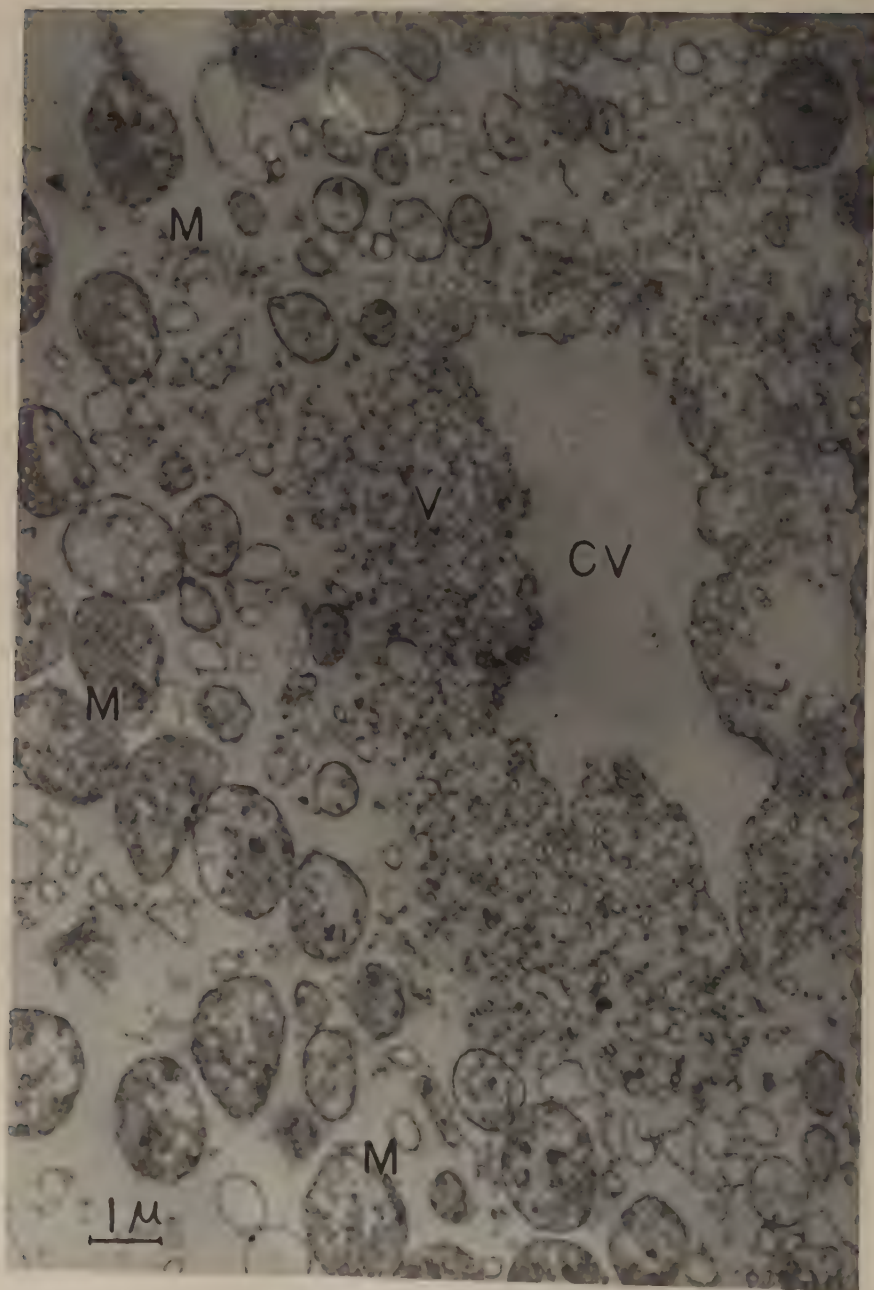


FIGURE 14. Electron micrograph of a portion of a contractile vacuole (CV) of *Pelomyxa carolinensis*. The contractile vacuole wall is surrounded by a layer of vesicles (V); these in turn are surrounded by mitochondria (M). $\times 9,200$.

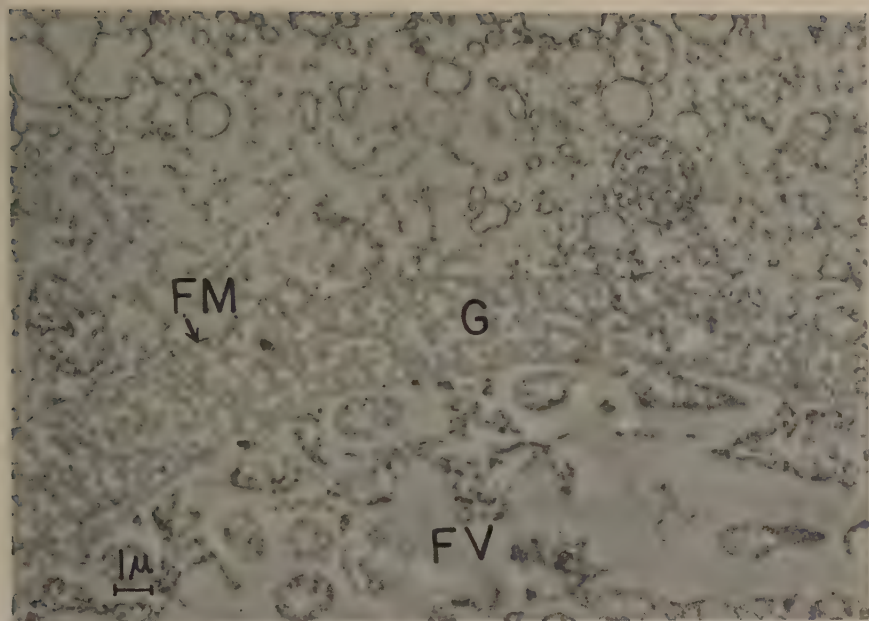


FIGURE 15. Electron micrograph showing a portion of a newly formed food vacuole (FV) in *Pelomyxa carolinensis*. A layer of granular material (G) is seen in close association with the food vacuole membrane (FM). This layer of granular material is characteristic of newly formed food vacuoles. $\times 4,600$.

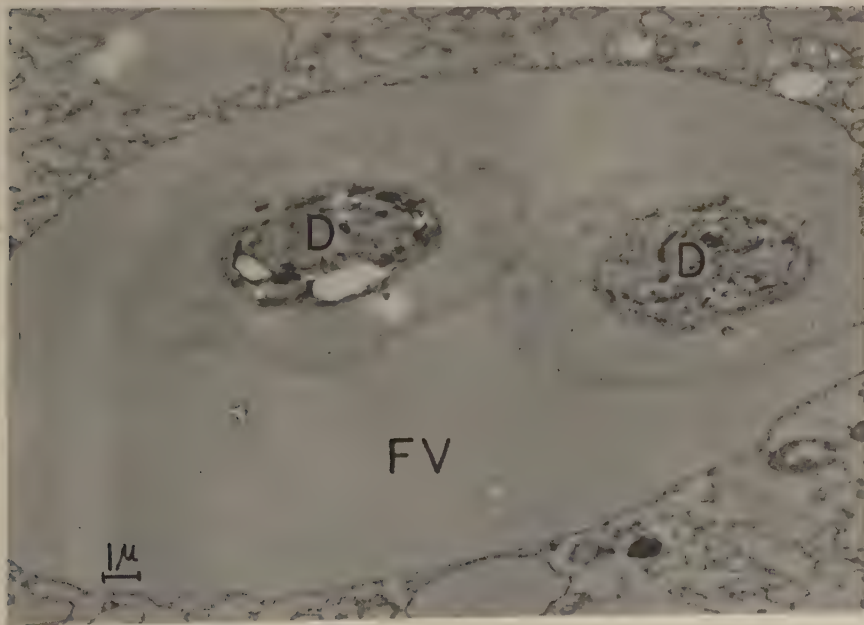


FIGURE 16. A portion of an old food vacuole (FV) containing two dense bodies (D) surrounded by fluid. $\times 4,600$.

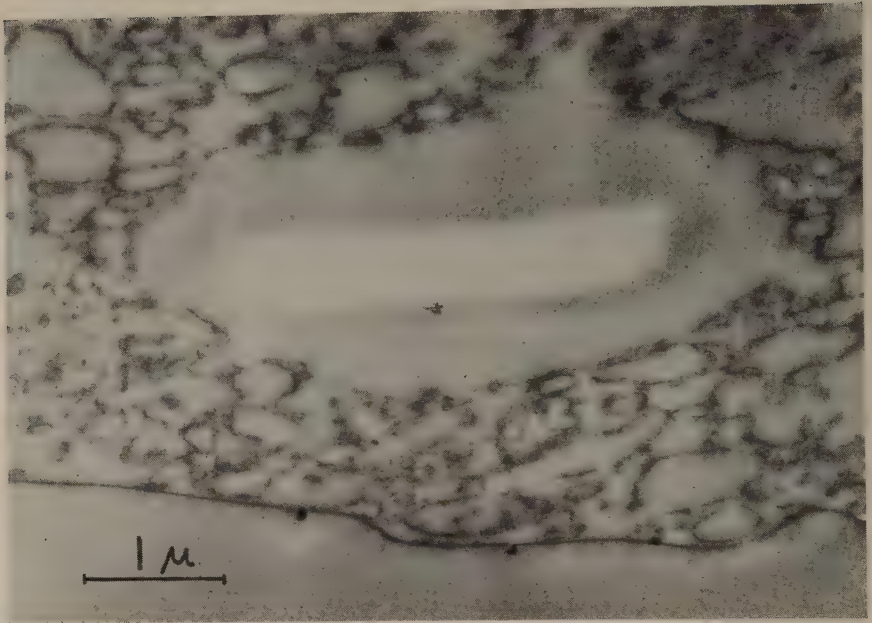


FIGURE 17. Electron micrograph of a portion of a crystal vacuole in *Pelomyxa carolinensis*. A clear area seen in the vacuole probably contained a platelike crystal. $\times 18,500$.

can be readily identified by the absence of embedding material in the space that formerly contained the crystal. Thus negative images are obtained. Negative images corresponding to the two types of crystals present in the living amoebae are found: platelike (FIGURE 17) and truncated bipyramidal (FIGURE 18).

Nuclear envelope. Pores formed by the two nuclear membranes can be seen readily in a tangential section through the nuclear envelope of *A. proteus* (FIGURE 19). Just within the nuclear membranes and adjacent to the nuclear pores, an inner honeycomb structure can be found. This structure may be described as an intraperinuclear network. The hexagonal form of the network components in *A. proteus* is characteristic of this species. The nuclear pores are about 640 \AA in diameter, while the inner hexagons are about 1400 \AA wide.

The nuclear envelope of *P. carolinensis* also contains pores formed by the two nuclear membranes (FIGURES 20 and 21). However, the honeycomb network found just within the nuclear membranes in *A. proteus* is not present in *P. carolinensis*. There is, instead a loose network of fibrils in the intraperinuclear area (FIGURES 20 and 22). The thickness of the fibrils making up this network is about 60 to 70 \AA . The depth of the intraperinuclear fibrillar network varies greatly (FIGURE 22).

The nuclear envelope of *H. rysodes* differs from that of *A. proteus* and *P. carolinensis* in that it does not contain any intraperinuclear structure. The two nuclear membranes of *H. rysodes* do form pores however, and these pores

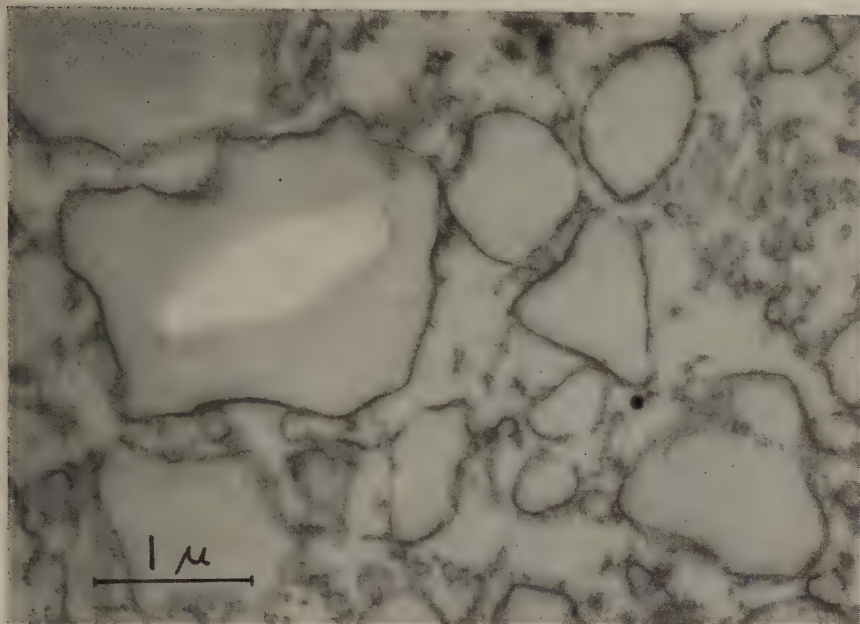


FIGURE 18. A portion of a crystal vacuole that had contained a truncated bipyramidal crystal. The crystal in this figure and in the preceding one presumably evaporated when the section was placed in the electron beam of the microscope, thus leaving a negative image. $\times 20,000$.

can be seen in a tangential view of the nuclear envelope (FIGURE 8). When the nuclear envelope is seen in cross section (FIGURE 8), small particles corresponding to those of the endoplasmic reticulum are found in contact with the cytoplasmic surface of the outer nuclear membrane.

Many nucleoli are found just within the nuclear envelope in *A. proteus* and *P. carolinensis* (FIGURES 19, 20, and 22).

Helices. In the inner areas of the nucleoplasm of both *A. proteus* and *P. carolinensis*, clusters of helices are found (FIGURES 22 and 23). The thickness of the filament making up the coil is about 120 to 130 Å. When a helix is seen in cross section (at arrow in FIGURE 22), it appears as a "doughnut" having a diameter of 350 Å.

The length of these coiled filaments varies greatly, the longest ones being about 2400 Å.

Discussion

Lehmann *et al.* (1956) and Manni (1956) have described the plasmalemma as a two-layered structure. Brandt (1958) demonstrated with the light microscope that an outer plasmalemmal "layer" may split off during fixation. Undoubtedly this outer layer represents the filamentous extensions shown in an earlier paper (Pappas, 1956b), and in FIGURES 1 to 3, and 5. The fine filaments

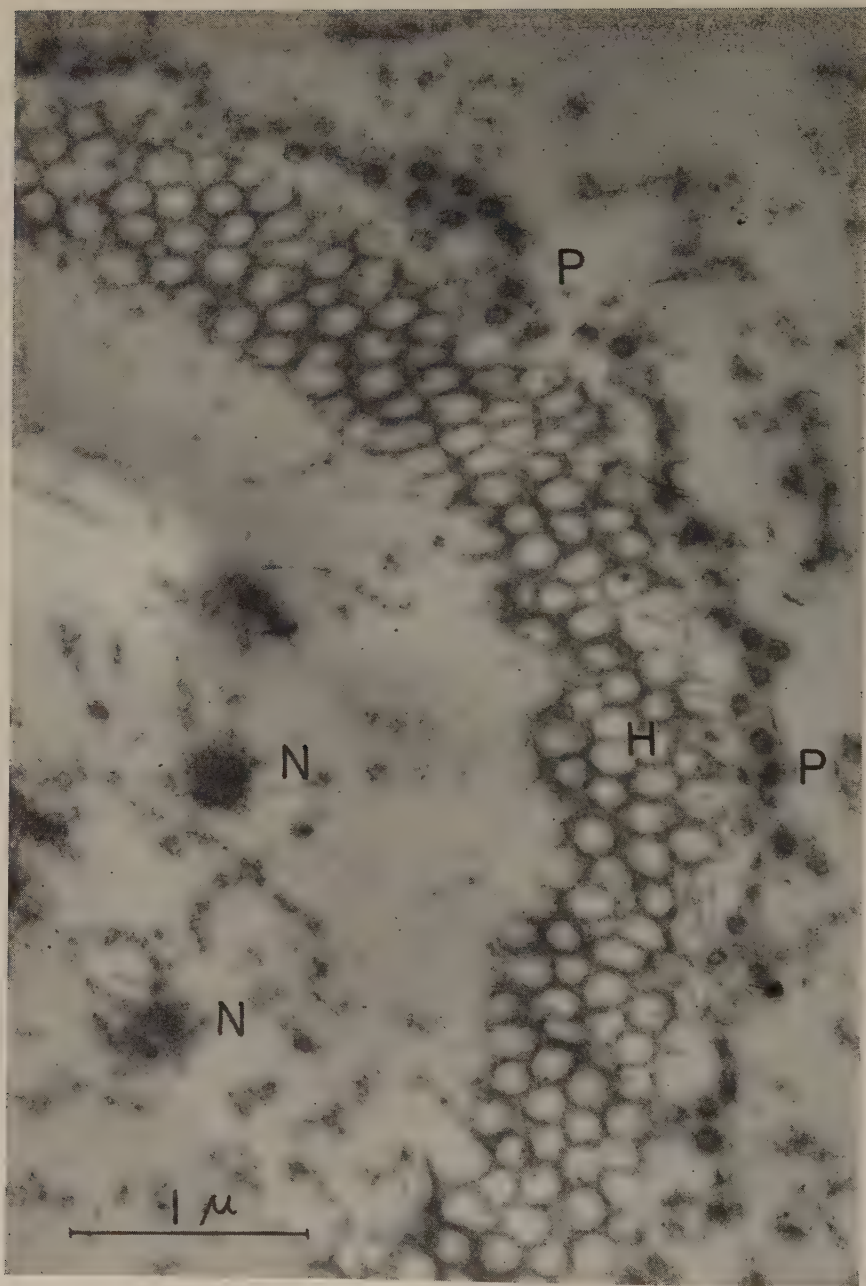


FIGURE 19. Electron micrograph of a tangential section through a portion of the nuclear envelope of *Amoeba proteus*. Pores (*P*) formed by the two nuclear membranes can be seen. The diameter of the pores is approximately 640 Å. A honeycomb structure (*H*) is found adjacent to the nuclear pores in the intraperinuclear area. The structures are about 1400 Å wide. Nucleoli (*N*) can be seen within the nuclear envelope. $\times 32,600$.

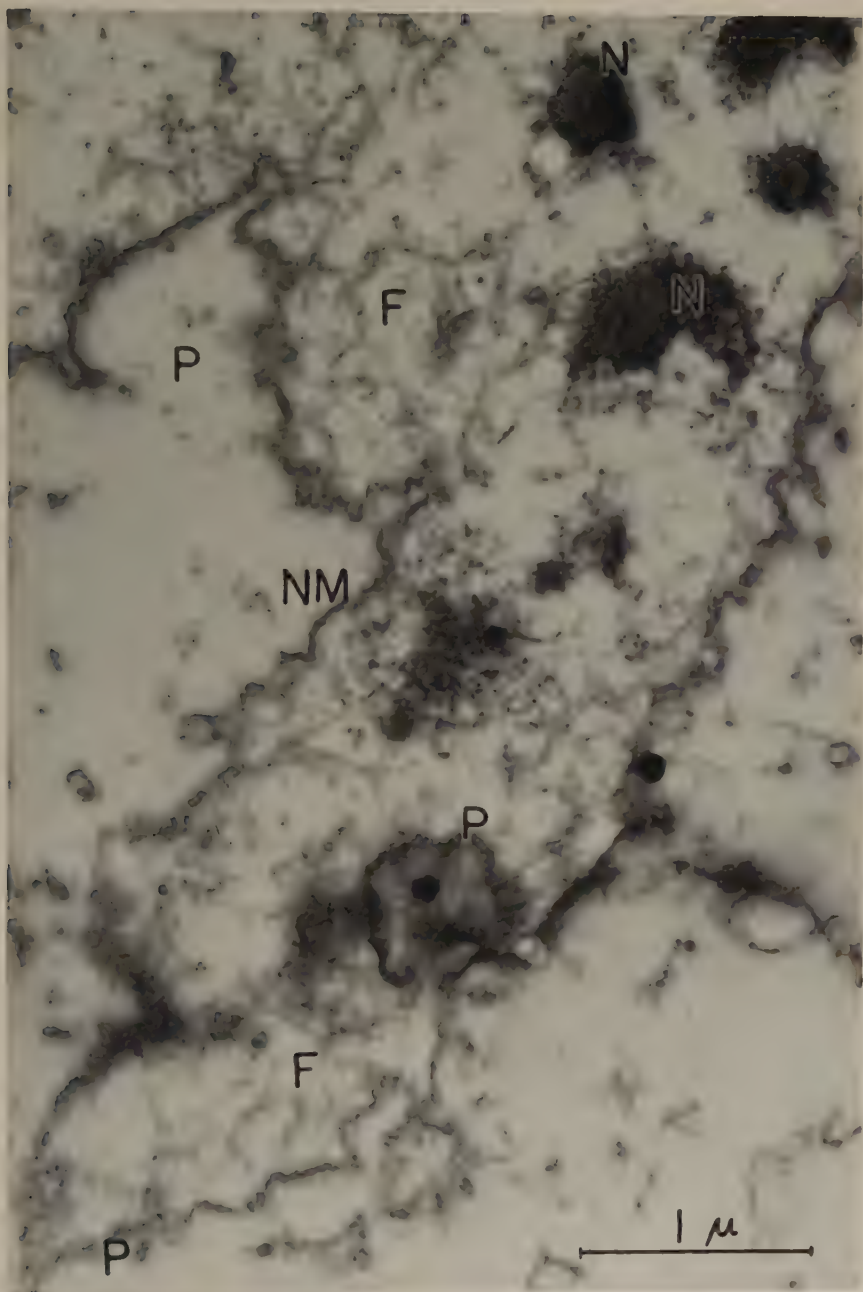


FIGURE 20. Electron micrograph through a portion of the nuclear envelope of *Pdelomyia carlinipennis*. When the two nuclear membranes (NM) are seen in tangential view, pores (P) formed by these membranes can be seen. The diameter of the pores is about 600 Å. A loose network of fibrils (F) is found within the nuclear membranes in the intraperinuclear area. The diameter of the fibrils making up this network is about 60 to 70 Å. Some nucleoli (N) can be seen in the nucleus. $\times 30,300$.

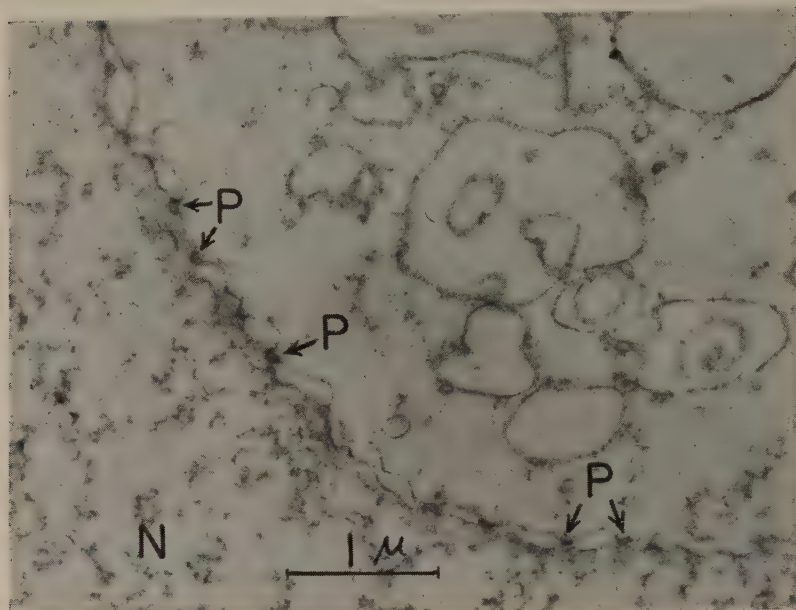


FIGURE 21. A slightly oblique section of a nuclear envelope of *Pelomyxa carolinensis*, showing pores (*P*) formed where the inner and outer membranes of the nucleus (*N*) are joined with one another. $\times 20,000$.

may serve as sites of adsorption of materials from the surrounding milieu. The structure of the plasmalemma, with its hairlike affiliates, provides an extended area for adsorption, although not increasing the membrane-cytoplasm interface area.

Increase of surface membrane area in metazoan cells is achieved in several ways. Infoldings of the cell membrane can produce this increase as, for example, in the basal portion of the proximal convoluted tubule cells of the kidney (Rhodin, 1954); alternatively, the increase in area can be due to the formation of fingerlike projections, such as the microvilli found in the apical surface of the choroid plexus (Maxwell and Pease, 1956; Pease, 1956). The increase of cell surface by infoldings or villi allows for a greater area of membrane-cytoplasm interface. The fine structure of the plasmalemma demonstrates still another type of specialization of the cell surface: an increase of the sites or loci for adsorption of materials from the surroundings, without increase of membrane-cytoplasm interface.

The alveolar structured appearance of the cytoplasm of *A. proteus* and *P. carolinensis* is unique among protozoan and metazoan cells studied to date with the electron microscope. While the preparation of the amoebae and the subsequent treatment of sections in the electron microscope may have produced this type of foamy cytoplasm, it should be noted that *H. rysodes* was prepared in identical fashion, yet does not have a comparable elaborate alveolar cytoplasm.

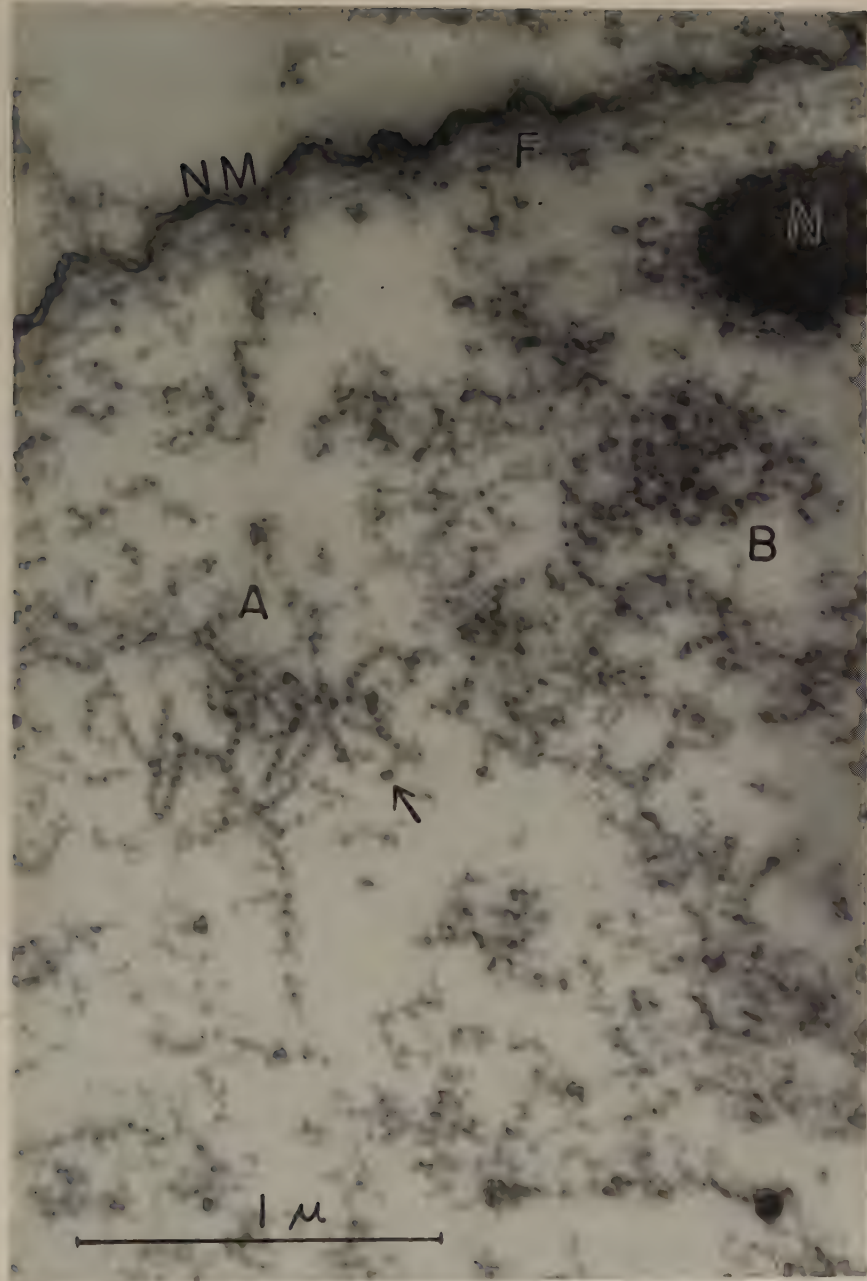


FIGURE 22. Electron micrograph of a portion of a nucleus of *Pelomyxa carolinensis*. The nuclear envelope made up of two membranes (NM), and an inner fibrillar network (F) are seen. The fibrillar network in the intranuclear area is about 1400 Å thick. A single nucleolus (N) is found just within the nuclear envelope. Clusters of helices are found within the nucleus at A. A doughnut-shaped cross section of a single helix is seen at the arrow. The size of these coiled structures is the same as those shown in figure 23. Material seen at B is similar to that shown at A. Only a few coils can be seen, due perhaps to the plane of sectioning. $\times 48,000$.

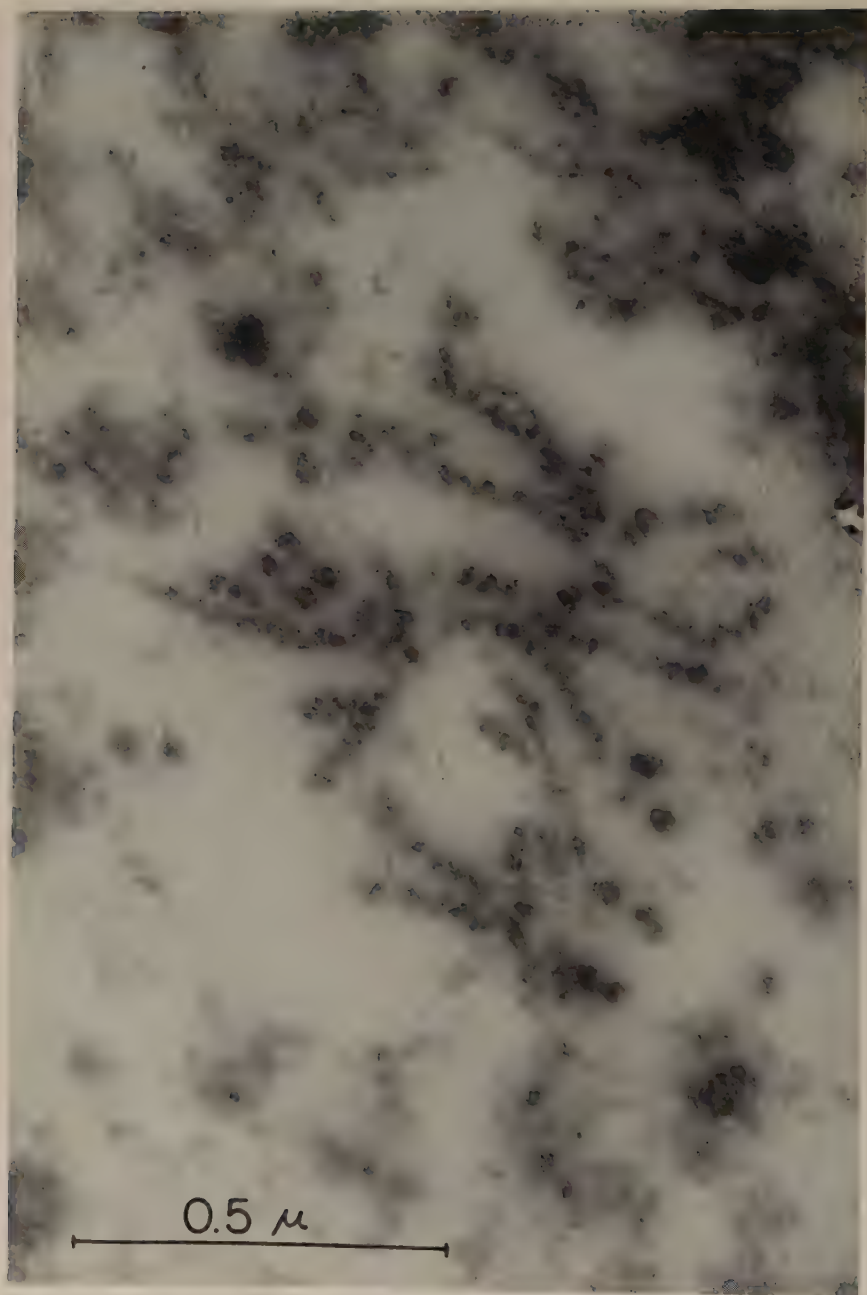


FIGURE 23. Electron micrograph of a portion of the nucleus of *Amoeba proteus*, showing clusters of helical structures. The filaments making up the coils are about 120 Å in thickness. The diameter of the coils is about 320 Å. The length of the coils cannot be accurately measured, since the apparent length varies, depending on the plane of sectioning. The longest coil measures about 2400 Å. $\times 100,000$.

Electron micrographs indicate that the basophilic component of the cytoplasm of metazoan cells is a network of flattened vesicles or cisternae and canaliculae, found in association with granules containing RNA-protein (Palade and Porter, 1954; Palade, 1956; Palade and Siekevitz, 1956). This type of structure is seen in *H. rysodes* (FIGURES 4, 7, and 8). The granular component of the endoplasmic reticulum is not abundant in the other two species of amoebae. The granules in *A. proteus* and *P. carolinensis* are present in small numbers per unit area and are distributed in whorls, similar to those described by Palay and Palade (1955) in neurons. The small number of granules per unit area in a highly hydrated cytoplasm probably accounts for the very diffuse and slight basophilia of the amoebae (Roskin and Ginsburg, 1944; Brachet, 1950; Pappas, 1954).

The patterns formed by the elaboration of infoldings and projections of the inner of the two mitochondrial membranes is striking. It is postulated that the profiles of mitochondria *A*, *B*, and *C* seen in FIGURE 11, are formed by a similar basic pattern. The different appearances of the pattern are due to sectioning in different planes. In order to define precisely the basic pattern of these mitochondria, a study of serial sections is now in progress. Our preliminary results indicate that the inner mitochondrial membrane has regularly arranged infoldings or cristae (Palade, 1952), in addition to the more commonly noted fingerlike projections or villi (Dalton and Felix, 1957). The villi either penetrate through the cristae in a regular sequence, or lie between the cristae, again in a regular sequence. When they penetrate the cristae, a cross section reveals doughnut-shaped structures (see mitochondrion *A* in FIGURE 11). The outer membrane of the doughnut is the limiting membrane of the cristae; the inner membrane is that of the villus.

A profile of a mitochondrion showing villi lying between cristae is seen in FIGURE 13. The regular sequence of the villi (shown in cross section) causes the surface of the cristae to become wavy.

Not all parts of the mitochondrion show infoldings of the inner membrane (see *S* in FIGURES 11 to 13). The area without infoldings contains most of the material of the stroma. The stroma or area in the mitochondrion within both membranes usually appears to be granular. However, the stromal material viewed in a longitudinally cut section appears as discrete oriented fibrils about 40 Å thick (FIGURE 12).

The stroma of mitochondria in *H. rysodes* is extremely dense (FIGURE 4). A similar dense stroma has been described (Cohen, 1957*b*) in mitochondria of starved *A. proteus*. The mitochondria in *Hartmannella* (FIGURE 4) are in close contact with lipid bodies. Palade and Shidlovsky (1958) have recently described similar close contact of lipid bodies and mitochondria in pancreatic and hepatic cells. They concluded that this association is either an expression of a shift from carbohydrate to lipid as the main source of cellular energy, or that active lipid synthesis is in process. Observations during various times in the growth phase of a culture of *Hartmannella* may shed more light on the relationship of mitochondria and lipid bodies.

The small vesicles surrounding the contractile vacuole appear to empty their contents into the vacuole and, at the same time, contribute their mem-

branes to the vacuole wall during diastole (FIGURE 10). No fibrillar components could be found (Bairati and Lehmann, 1956; Lehmann *et al.* 1956) in the area of the contractile vacuole. The vesicles surrounding the contractile vacuoles may be derived from pinocytosis vacuoles or components of the endoplasmic reticulum. In FIGURE 10, at C, small vesicles in chainlike linear fashion are seen, suggesting a break down of a single larger structure. A mechanism for the segregation of water from the cytoplasm into the contractile vacuole, using pinocytosis as a model, has been suggested (Pappas and Brandt, 1958). It is postulated that the small vesicles surrounding the contractile vacuole are the loci of solute-solvent segregation.

In an electron microscope study, Gatenby *et al.* (1955) suggested that the contractile vacuole in protozoa is analogous to the Golgi apparatus. In amoebae, arrays of flattened vesicles packed very closely together (Cohen, 1957a; also FIGURES 6, 9, and 10) bear a striking resemblance to the Golgi complex of metazoan cells (Dalton and Felix, 1956; Pollister and Pollister, 1957). Similar structures have been described in ciliated (Noirot-Timotheé, 1957) and in flagellated protozoa (Rouiller and Fauré-Fremiet, 1958). The contractile vacuole and its components, on the other hand, bear no morphologic resemblance to the metazoan Golgi complex.

The close association of the granular material of a newly formed food vacuole with the food-vacuole membrane may be related to digestion. In older food vacuoles, such associations are not found. A study of the changes seen as a newly formed food vacuole progresses to an old vacuole will be useful in our understanding of the process of food-vacuole digestion.

The clear outlines produced by the negative images of crystals suggest that the crystal material evaporates in the electron beam. It seems unlikely that the crystals fall out during sectioning, since then they would most likely cause scratching in the section.

In the present study, spherical refractive bodies and alpha granules were not identified. Cohen (1957a) has recently described some particles in vacuoles as alpha particles.

The nuclear envelope of *A. proteus* has been described previously as a double membrane that contains pores, 640 Å in diameter. It is believed that the pores are formed when the inner and outer membranes are joined with one another (Pappas, 1956b). The nuclear envelopes of *P. carolinensis* and *H. rysodes* also contain pores of the same size (FIGURES 8 and 20). Since no direct evidence exists that the nuclear pores are patent, some investigators have found the term "annuli" more appropriate (Greider *et al.*, 1956; Rebhun, 1956; Gall, 1956; Swift, 1956). In the three species of amoebae, the pores occupy about 15 to 20 per cent of the nuclear surface, almost twice the amount estimated by Watson (1955) for mammalian cells.

The function of the pores is not yet fully known. Since rapid transfer of nuclear ribonucleic acid into the cytoplasm has been demonstrated recently by Goldstein and Plaut (1955) in *A. proteus*, it is interesting to speculate that the pores may provide one pathway for the rapid transfer of materials into and out of the nucleus.

The nuclear envelope of *A. proteus* contains an additional structure consisting of a thick layer of closely packed hexagonal prisms, each prism terminating at the nuclear envelope in a precisely centered pore (Pappas, 1956b). In the intraperinuclear space of *P. carolinensis*, a loose network of fibrils, rather than hexagonal prisms, is present. *H. rysodes* on the other hand, does not contain any intraperinuclear structures. The function of these nuclear structures remains obscure. It has been suggested that the honeycomb structure of *A. proteus* disappears during nuclear division (Cohen, 1957a).

Helical structures have been found in the nuclei of both *A. proteus* and *P. carolinensis*. The filaments making up the coiled structures in FIGURES 22 and 23 are about 120 to 130 Å thick. The diameter of the coils is about 300 to 350 Å. In the first report of helices in *A. proteus*, the filaments of the coils were reported as almost half of this size, that is, 70 Å (Pappas, 1956a). It appears that the 120 to 130 Å filaments are not single unit structures, particularly in view of evidence indicating splitting in some of the larger coils. These data will be published subsequently.

Feulgen preparations of amoebae have shown that the inner portion of the nuclei is diffusely Feulgen positive. This is the area in which helices are found. The helices occupy an intermediate position in size between the chromatin material and the DNA molecule. Studies are now in progress on the appearance and distribution of helices during the stages of nuclear division.

Summary

The fine structure of three species of amoebae was studied: *Amoeba proteus*, *Pelomyxa carolinensis* (*Chaos chaos*), and *Hartmannella rysodes*.

The plasmalemma of *A. proteus* and *P. carolinensis* has fine fibrous extensions on its outer surface. The structure of the plasmalemma with its hairlike affiliates provides an extended area for adsorption, although not increasing the membrane-cytoplasm interface area.

The endoplasmic reticulum was studied in the three species of amoebae. The granular component of the endoplasmic reticulum is not abundant in the alveolar-structured cytoplasm of *A. proteus* and *P. carolinensis*.

In the three species of amoebae studied, arrays of flattened vesicles packed very closely together bear a striking resemblance to the Golgi complex of metazoan cells. The structure of the mitochondria appears similar to that described in other protozoa. However, some mitochondria found in *P. carolinensis* have a more complex organization, producing strikingly complex patterns formed by the inner mitochondrial membrane. In addition, fine filaments are found in the stroma of mitochondria.

The contractile vacuole is surrounded by a densely packed layer of small round vesicles. The vesicles in turn are surrounded by mitochondria. The vesicles appear to empty their contents into the vacuole and, at the same time, to contribute their membranes to the vacuole wall during diastole.

The close association of the granular material of newly formed food vacuoles with the vacuole membrane may be related to digestion.

The clear outlines produced by the negative images of crystals suggest that the crystal material evaporates in the electron beam.

Just within the two nuclear membranes and adjacent to the pores found by these membranes, an inner honeycomb structure is found in *A. proteus*. In *P. carolinensis* the honeycomb network is not found, but there is a loose network of fibrils in the intraperinuclear area. Neither honeycomb nor loose fibrils are found in the intraperinuclear area of *H. rhyodes*.

Clusters of helical structures are found in the inner parts of the nuclei of *A. proteus* and *P. carolinensis*.

References

- ANDRESEN, N. 1956. Cytological investigations on the giant amoeba *Chaos chaos* L. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **29**: 435.
- BAIRATI, A. & F. E. LEHMANN. 1956. Structural and chemical properties of the contractile vacuole of *Amoeba proteus*. *Protoplasma*. **45**: 525.
- BRACHET, J. 1950. The localization and the role of ribonucleic acid in the cell. *Ann. N. Y. Acad. Sci.* **50**(8): 861.
- BRANDT, P. W. 1958. A study of the mechanism of pinocytosis. *Exptl. Cell Research*. **15**: 300.
- COHEN, A. I. 1957a. Electron microscopic observations of *Amoeba proteus* in growth and inanition. *J. Biophys. Biochem. Cytol.* **3**: 859.
- COHEN, A. I. 1957b. The cell in starvation: physiological and chemical observations. *J. Biophys. Biochem. Cytol.* **3**: 923.
- DALTON, J. A. & M. D. FELIX. 1956. A comparative study of the Golgi complex. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 79.
- DALTON, J. A. & M. D. FELIX. 1957. Electron microscopy of mitochondria and the Golgi complex. *Symposium Soc. Exptl. Biol.* **10**: 148.
- GALL, J. G. 1956. Small granules in the amphibian oocyte nucleus and their relationship to RNA. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 393.
- GATENBY, J. B., A. J. DALTON & M. D. FELIX. 1955. The contractile vacuole of Parazoa and Protozoa and the Golgi apparatus. *Nature*. **167**: 301.
- GREIDER, M. H., W. J. KOSTIR & W. J. FRAJOLA. 1956. Electron microscopy of the nuclear membrane of *Amoeba proteus*. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 445.
- GOLDSTEIN, L. & W. PLAUT. 1955. Direct evidence for nuclear synthesis of cytoplasmic ribose nucleic acid. *Trans. Natl. Acad. Sci.* **41**: 874.
- LEHMANN, F. E., E. MANNI & A. BAIRATI. 1956. Der Feinbau von Plasmalemma und Kontraktiler Vakuole bei *Amoeba proteus* in Schnitt- und Fragment- präparaten. *Rev. suisse Zool.* **63**: 246.
- LEHMANN, F. E., E. MANNI & W. GEIGER. 1956. Der Schichtenbau des Plasmalemmas von *Amoeba proteus* in elektronenmikroskopischen Schnittbild. *Naturwiss.* **43**: 91.
- MANNI, E. 1956. Ricerche sulla struttura submicroscopica dell *Amoeba proteus*. I. Plasmalemma, nuleo. *Boll. soc. ital. biol. sperm.* **32**: 113.
- MAXWELL, D. S. & D. C. PEASE. 1956. The electron microscopy of the choroid plexus. *J. Biophys. Biochem. Cytol.* **2**: 467.
- NOIROT-TIMOTHÉE, C. 1957. L'ultrastructure de l'appareil de Golgi des Infusoires Ophryoscolecidae. *Comp. rend.* **244**: 2847.
- PALADE, G. E. 1952. The fine structure of mitochondria. *Anat. Record*. **114**: 427.
- PALADE, G. E. 1955. A small particulate component of the cytoplasm. *J. Biophys. Biochem. Cytol.* **1**: 59.
- PALADE, G. E. 1956. The endoplasmic reticulum. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 85.
- PALADE, G. E. & K. R. PORTER. 1954. Studies on the endoplasmic reticulum. I. Its identification in cells *in situ*. *J. Exptl. Med.* **100**: 641.
- PALADE, G. E. & P. SIEKEVITZ. 1956. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* **2**: 171.
- PALADE, G. E. & G. SHIDLOVSKY. 1958. Functional association of mitochondria and lipid inclusions. *Anat. Record*. **130**: 352.
- PALAY, S. L. & G. E. PALADE. 1955. The fine structure of neurons. *J. Biophys. Biochem. Cytol.* **1**: 69.

- PAPPAS, G. D. 1954. Structural and cytochemical studies of the cytoplasm in the family *Amoebidae*. *Ohio J. Sci.* **54**: 195.
- PAPPAS, G. D. 1956a. Helical structures in the nucleus of *Amoeba proteus*. *J. Biophys. Biochem. Cytol.* **2**: 221.
- PAPPAS, G. D. 1956b. The fine structure of the nuclear envelope of *Amoeba proteus*. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 431.
- PAPPAS, G. D. & P. W. BRANDT. 1958. The fine structure of the contractile vacuole in amoeba. *J. Biophys. Biochem. Cytol.* **4**: 485.
- PEASE, D. C. 1956. Infolded basal plasma membranes found in epithelia noted for their water transport. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 203.
- POLLISTER, A. W. & P. F. POLLISTER. 1957. The structure of the Golgi apparatus. *Intern. Rev. Cytol.* **6**: 85.
- POWERS, E. L., C. F. EHRET, L. E. ROTH & O. T. MINICK. 1956. The internal organization of mitochondria. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 341.
- REBHUN, L. I. 1956. Electron microscopy of basophilic structures of some invertebrate oocytes. I. Periodic lamellae and the nuclear envelope. *J. Biophys. Biochem. Cytol.* **2**: 93.
- RHODIN, J. 1954. Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. *Aktiebolaget Goduil*. Stockholm, Sweden.
- ROSKIN, G. I. & A. S. GINSBURG. 1944. Zymonucleic acid of the protozoic cell. *Compt. rend. acad. sci. U.R.S.S.* **42**: 348.
- ROUILLER, C. & E. FAURÉ-FREMIET. 1958. Structure fine d'un flagelle crysomonadien *Chromulina psammobia*. *Exptl. Cell Research.* **14**: 47.
- RUDZINSKA, M. A. & K. R. PORTER. 1954. The fine structure of *Tokophrya influsioenum* with emphasis on the feeding mechanism. *Trans. N. Y. Acad. Sci.* **16**(8): 408.
- SEDAR, A. W. & K. R. PORTER. 1955. The fine structure of cortical components of *Paramecium multimicronucleatum*. *J. Biophys. Biochem. Cytol.* **1**: 583.
- SEDAR, A. W. & M. A. RUDZINSKA. 1956. Mitochondria of protozoa. *J. Biophys. Biochem. Cytol.* **2** (Suppl.): 331.
- SWIFT, H. 1956. The fine structure of annulate lamellae. *J. Biophys. and Biochem. Cytol.* **2** (Suppl.): 415.
- WATSON, M. L. 1955. The nuclear envelope. Its structure and relation to cytoplasmic membranes. *J. Biophys. Biochem.* **1**: 257.
- WOLKEN, J. J. & G. E. PALADE. 1953. An electron microscope study of two flagellates. Chloroplast structure and variation. *Ann. N. Y. Acad. Sci.* **56**(5): 873.

PELOMYXA AND RELATED ORGANISMS

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It seems to be a matter of common knowledge that there exists a discord with respect to the nomenclatural and generic status of various amoebae. This discord came into being when pioneer microscopists found and named many amoeboid organisms without adequately describing them. The result was that no one could determine what others had seen. I need not recount here the state of nomenclatural confusion during the early days, since it had been dealt with by several authors. I shall discuss briefly how the matter stands in recent years regarding the nomenclature of amoebae.

Nomenclature

It began when Rösel von Rosenhof (1755) discovered an organism and wrote about it under the name of "der kleine *Proteus*." Linnaeus (1758) named it *Volvox chaos* and then renamed it *Chaos proteus* (1767). As *Volvox* is considered a protozoan by the majority of protozoologists, the binomial name of Rösel's "kleine *Proteus*" is *Chaos chaos* L. Beginning with O. F. Müller (1786), who saw and named *Proteus diffluens*, many microscopists found numerous amoeboid organisms between 1786 and 1878. As to what these amoebae are, diverse views appeared. Leidy (1878, 1879), in describing *Amoeba proteus* for the first time adequately, listed both *Chaos chaos* and *Proteus diffluens* as its synonyms. Stiles (1905) and Stiles and Boeck (1923) maintained that *Chaos chaos* was generically and specifically like *Amoeba proteus* (Leidy, 1878) without presenting any valid reason for holding such a view. Schaeffer (1916) first agreed with Leidy and Stiles in assuming the identity of *Chaos chaos* and *Amoeba proteus*, but ten years later changed his view in his "preliminary system" of classification of the amoebae (1926) and came to believe that Müller's *Proteus diffluens* was the same as *Amoeba proteus* and that Rösel's "kleine *Proteus*" was none other than *Pelomyxa carolinensis* described and named by Wilson in 1900. Schaeffer therefore advocated that the valid names of *Pelomyxa carolinensis* and *Amoeba proteus* are *Chaos chaos* L. and *C. diffluens* (M.), respectively.

Mast and Johnson (1931) studied Rösel's observation and presented evidence to show that *Chaos chaos* is "neither generically nor specifically like either Leidy's *proteus* or Wilson's *carolinensis*," but that "it is in fact a myxomycete," and that Schaeffer's contention that *C. chaos* is identical with *P. carolinensis* is false. Rice (1945) also disagreed with Schaeffer's assumption by pointing out that the diversity of opinion as stated above is in itself a strong support for the contention that reasonable proof of the identity of *C. chaos* cannot be found, and maintained that *P. carolinensis* is the valid name for Wilson's organism. Some years ago, I examined Rösel's paper in the light of my observations on *P. carolinensis* and *P. illinoisensis*, and concluded that it is not possible to determine the identity of *Chaos chaos* and that Schaeffer's assumption is not supported by facts (Kudo, 1946, 1952). Recently I have had the good fortune to

observe the oldest known species of *Pelomyxa*, namely *P. palustris*, in culture. With the added data from this source, I shall dwell upon the subject once more.

In arriving at his assumption that *Chaos chaos* is identical with *Pelomyxa carolinensis*, Schaeffer (1926) stresses 4 points in Rösel's observation as possessing "positive determining value." First, he places great confidence in Rösel as a reliable investigator and a very able draughtsman. Rösel included in his paper 19 drawings, one of which (FIGURE 1A) shows a spherical organism that was said to represent its natural size; it measures about 1.5 mm. in diameter. The magnification of the other 18 figures is not given, but his FIGURE 1B measures about 9 mm. in diameter; therefore, by comparison with FIGURE 1A, it was magnified about 6 times its natural size. FIGURES 1C to T were probably

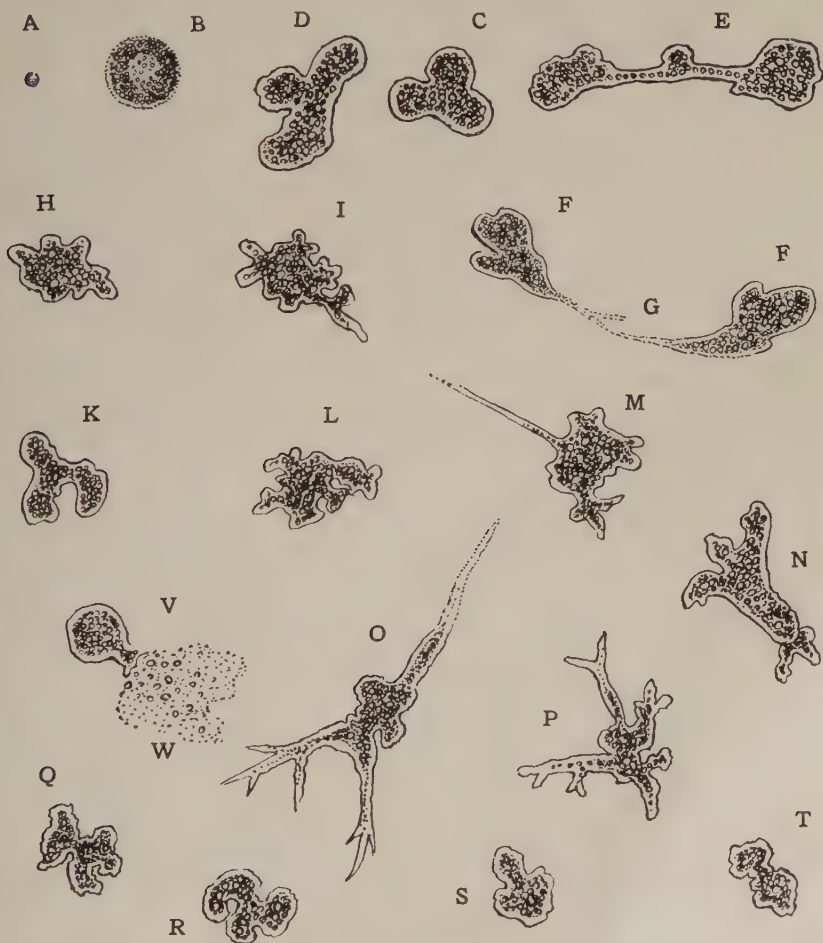


FIGURE 1. Line drawing reproduction of the 19 figures of "der kleine *Proteus*" by Rösel von Rosenhof (1755). The granulated area in each figure is colored yellow in the original. Approximately the same size as the original plate.

similarly magnified. With such a low magnification, even external morphologic features, not to mention internal structure, cannot be depicted accurately.

Second, Schaeffer considers the size of amoebae the most readily applied specific test; with this view all agree. As examples of large amoebae, he listed 3 species of amoeba and 2 species of *Pelomyxa*, and then stated that "if the size is taken into consideration also, Rösel's description fits only one species now known, the *carolinensis* of Wilson, and so far as it goes, it fits it completely." This, however, is not the case, since all 3 species of *Pelomyxa* (*P. palustris*, which is the largest, *P. carolinensis*, and *P. illinoisensis*, which was, of course, unknown to Schaeffer) would in rounded forms approximate the size of *Chaos chaos*. Furthermore, an important point of which Schaeffer was unaware is the fact that the spherical form shown by Rösel in his FIGURE 1B has a smooth contour, a condition not found in *carolinensis* or *illinoisensis* except in degenerating individuals, but one commonly found in spherical forms of *palustris*. This particular specimen (FIGURE 2B) seen by Rösel was apparently not degenerating, as he saw it changed into various forms (FIGURE 2C and D). Therefore, in the size, form, and appearance of the spherical form, *C. chaos* resembles more closely *P. palustris* than either *P. carolinensis* or *P. illinoisensis*.

Third, Schaeffer observes that only *A. proteus*, *A. nobilis*, and *P. carolinensis* form pseudopods that are antlerlike in appearance, as shown by Rösel in his figure (FIGURE 1-O) that depicts two long branching projections with sharply pointed ends. Mast and Johnson (1931) have already called attention to the fact that such pseudopods are "very rarely if ever" found in *A. proteus* or *P. carolinensis*, but they are frequently seen in the plasmodia of certain mycetozoa. Granted that the low magnification available to Rösel may have led him to draw sharply pointed pseudopods that actually might have been bluntly rounded, reference must be made not only to *P. carolinensis*, but also to other forms such as *P. illinoisensis*.

Fourth, Schaeffer wrote that "*Pelomyxa palustris* does not, probably can not move around freely on the vertical sides of a glass," in referring to such movements reported by Rösel for *C. chaos*. In this Schaeffer is absolutely in error. As early as 1894, Blochmann reported active vertical movements of *P. palustris*, which were also observed by Stolc (1900) and Leiner (1924). I myself have noticed it (Kudo, 1957). In fact, all three species of *Pelomyxa* are capable of locomotion on the vertical walls of glass containers.

As to the peculiar binary fission depicted by Rösel in his figures E to I, he wrote that a spherical form (FIGURE 2B) soon changed into a three-leaf clover form (FIGURE 2C) that changed further into the form shown in FIGURE 2D in less than half a minute. This form in turn became elongate. As the elongation continued (FIGURE 2E, F), it appeared as though the organism would divide into two parts. It actually did divide into two parts by separation at the point marked G. There were now two animals instead of one, and these assumed different body forms as indicated (FIGURE 2H and I). Such a division does not occur in *P. carolinensis* (Kudo, 1946, 1947, 1949) or in *P. illinoisensis* (Kudo, 1951; McClellan, 1954) in which nuclear and cytoplasmic divisions have been thoroughly studied.



FIGURE 2. Photographic reproduction of Rösels' FIGURES B to I, enlarged about 3 times.

It has been stated by Schaeffer that because of highly irregular and spotty occurrence of various amoebae in nature, the fact that *P. carolinensis* had not been found in Europe had no bearing on his interpretation. Rösel did not state where he collected his "kleine *Proteus*," but it was probably collected in Germany. *P. palustris*, first discovered in Marburg (Greeff, 1874), has been found to occur in various localities in Germany and neighboring countries, as well as in Great Britain and in the United States (Kudo, 1957), while *P. carolinensis* has so far not been found outside the United States. Here reference must be made to a brief note by McGuire (1932), who reported the presence of an amoeba similar to *P. carolinensis* observed by Kepner and Edwards (1917) in Great Britain but, in the absence of further information, its exact nature remains unknown. However, if this were *P. carolinensis*, it would appear that the chances of Rösel's finding *P. palustris* would have been far greater than finding *P. carolinensis*.

Rösel's drawings show very little internal structure (FIGURE 2). Except in the spherical form, there is a clear peripheral zone of uniform width, and the main body is filled with granules of different sizes. There is absolutely no cytological information of value for identifying Rösel's organism. In the opinion of Mast and Johnson (1931), if one considers the structure as a whole, it clearly resembles plasmodia of mycetozoa more closely than either *A. proteus* or *P. carolinensis*.

In support of his assumption, Schaeffer (1926) wrote: "The preceding discussion is not based merely on a succession of bibliographical references but is reinforced by actual study of the amebas themselves. I have myself observed and studied in the laboratory all of the species of amebas named above." We are greatly indebted to Schaeffer for his valuable contributions on the food habits and morphology of various amoebae. However, the records do not seem to support his statement, since one fails to find any indication among Schaeffer's published papers prior to 1926 that he studied *P. palustris*, or that he saw more than one individual of *P. carolinensis*. Thus it seems to me that the entire discussion given by him regarding the identity of Rösel's "kleine *Proteus*" and *P. carolinensis* was based upon information he obtained from the bibliographical references only.

I have shown here that none of the arguments Schaeffer has used to substantiate his assumption is supported by factual evidence, and that it is impossible to identify inadequately described organisms such as Rösel's "kleine *Proteus*" with well-defined species. Therefore, it is clear that *P. carolinensis* is the valid name of the organism described and named by Wilson (1900). *Chaos chaos* Linnaeus remains an unidentifiable organism of historical interest.

About ten years later, Schaeffer (1937, 1937a) found *P. carolinensis* in New Jersey and succeeded in obtaining laboratory cultures. He continued to call it *C. chaos* without further considering the validity of such usage and wholly ignoring Mast and Johnson's criticism (1931). These cultures under the name of *Chaos chaos*, were made available to schools and colleges by a supply company, and this erroneous name undoubtedly contributed much toward its popularity among lay students of protozoa. Mast (1938) pointed out again this

nomenclatural error, but to no avail. It is strange to note that among a number of investigators of *P. carolinensis*, which they designate *C. chaos*, not one person seems to have undertaken the task of examining Rösels's paper and Schaeffer's statement on 4 points of "positive determining value" to verify Schaeffer's assumption.

Let us now turn briefly to the nomenclature of *Amoeba proteus*. As mentioned before, Müller (1786) saw an amoeboid organism and named it *Proteus diffluens*. He attached to his statement 12 figures of probably one individual. These figures (FIGURE 3) show that the organism is capable of undergoing amoeboid form change with 3 or 4 more or less long pseudopods and somewhat granulated body protoplasm. It is said to have been many times smaller than Rösels's "kleine *Proteus*." Müller considered that it was probably a young *Proteus*. Unfortunately he failed to indicate any structural characteristic of the amoeba.

Stiles (1905) assumed that Müller's organism was an amoeba and named it *A. diffluens*, since he believed that *C. chaos* was *A. proteus*. Schaeffer (1926), on the other hand, supposed that *P. diffluens* was identical with *A. proteus*, and proposed to call it *C. diffluens*, as he believed it to be congeneric with *C. chaos*.

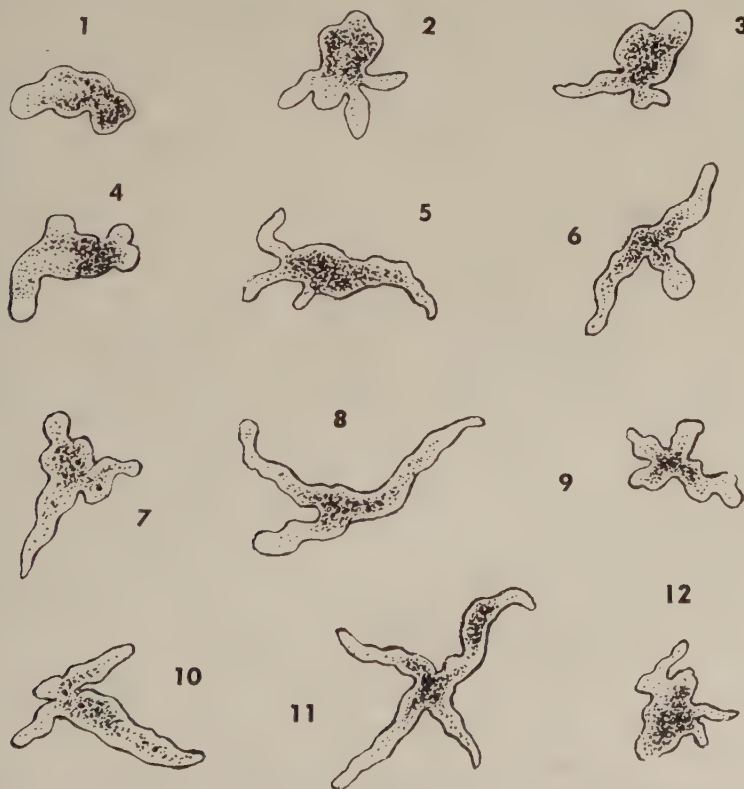


FIGURE 3. Line drawing reproduction of the 12 figures of *Proteus diffluens* by O. F. Müller (1786).

Neither Stiles nor Schaeffer offered any evidence to substantiate the reasonableness of his contention. The discrepancy of the views held by these two investigators in itself is a clear indication that incompletely described organisms cannot be identified. We are indebted to Leidy (1879) for his monumental work on sarcodinans, in which he gave adequate description of an amoeba that he named *A. proteus*. Schaeffer (1926) later redefined and differentiated it from *A. dubia* and *A. discoides*. As long as there is no positive proof that *A. proteus* had been seen before Leidy described it, this name remains valid.

Generic Characters in Amoebae

There is a diversity of views concerning what constitutes the generic characters in amoebae. Some of them will be discussed here.

External morphology. While there is no constant body shape in any of the amoebae, certain external peculiarities that are often found have been used as generic distinction. For instance, Fromentel (1874) created the genus *Trichamoeba* for an amoeba with filamentous processes. However, these processes vary in form and dimensions and are noticed in a variety of amoebae, appearing temporarily in connection with locomotion. Leidy (1875) established the genus *Dinamoeba* for an amoeba whose entire body surface was covered with minute spicules or rigid cils that were frequently absent and in several instances disappeared after having been present in large numbers. Thus these generic criteria lack a constant and precise definition.

By far the most elaborate subdivision of the genus *Amoeba* was undertaken by Schaeffer (1926), who described 39 new species and created 11 new genera based upon the external morphology: namely, the shape and character of pseudopods and types of locomotion that he thought were of primary importance. In his introduction Schaeffer wrote: "It would be a comparatively simple matter to adduce a number of instances in the study of amebas where conclusions too highly generalized were drawn from observation of only one species or from an inadequately described species." Nevertheless, he described in the very same paper three new species: *Trichamoeba caerulea*, *Metachaos rarum*, and *Dinamoeba horrida*, each from a single specimen, and *Metachaos oxyuris* from 2 specimens. Apparently he considered these specimens "normal," because he adjudged the normality of an amoeba by coordinate movement, by feeding or partially digested food materials, or by a division giving rise to daughters that undergo coordinate movement. He failed to take the population into account. Amoebae normally multiply in number in the presence of proper food and in optimal environment. Therefore, when one finds only one or two individuals after a long search, these may not be normal specimens. Whatever state an amoeba may be in, establishing a species based upon a single specimen is to me a very dangerous practice and must be avoided.

Schaeffer distinguished determinate and indeterminate pseudopods and stated that "no amoeba is known in which both pseudopods are present in typical form." I think that this is not the case, for both are often seen in typical forms of *P. carolinensis* or *P. illinoisensis*. Schaeffer's external morphologic features are based upon the habitual figures of amoebae without reference to defined environmental conditions such as temperature, light, pH, and composi-

tion of water. It is generally recognized that the pseudopods and, hence, the body form of an amoeba vary under different environmental and internal conditions. Ray and Hayes (1954) noted that in a small amoeba, *Hartmannella astronyxis*, various types of pseudopods are formed under different environmental factors. Unfortunately, there is no large free-living amoeba in which pseudopods, locomotion, and other morphologic characteristics have been made clear in relation to environmental or internal conditions. It follows, then, that what one observer sees in an amoeba as the normal external characteristics may or may not be noted as such by another observer examining the same organism under different circumstances.

To be acceptable for general use, a genus must be founded on distinctive diagnostic characteristics by which it is distinguished without difficulty from other genera by all competent workers. Let us examine the genera proposed by Schaeffer in 1926. *Trichamoeba* (Fromentel, 1874) is defined in part: "... by the possession during locomotion, of a uroid consisting of numerous, thin hair-like projections extending from the posterior end and by an elongate or clavate body shape." No precise definition of the form or dimensions of these hairlike projections is provided. If they are considered as the primary character of the genus, then *Metachaos oxyuris*, *Flabellula* spp., *Pelomyxa palustris*, and others would have to be placed in it.

Genus *Metachaos* is defined as "large amebas that undergo locomotion by means of determinate pseudopods, that is pseudopods that direct locomotion," which is contrary to what Schaeffer stated (page 7): "in no case, however, do these (determinate) pseudopods enlarge sufficiently so that the whole ameba flows into them; that is, they do not direct locomotion." "The general shape of the body is oblong, resembling an antler or staghorn" which, however, is not evident in the illustrations depicting the four new species he described. *M. oxyuris* is said to have "pseudopods few, blunt, indeterminate, directing locomotion." It appears that this species, together with *M. rarum* and *M. fulvum*, has a body form much different from *M. gratum*.

For the genus *Chaos*, Schaeffer writes: "The amebas belonging to this genus are large and form indeterminate pseudopods, that is, pseudopods that direct locomotion. The pseudopods are subcylindrical, blunt, filled with granular endoplasm throughout, and bear conspicuous longitudinal ridges and grooves in the ectoplasm. These grooves have tongues of ectoplasm extending down into the endoplasm so that the endoplasmic stream seems to be divided into smaller parallel streams. Often in locomotion when moving over a smooth surface the ameba takes on a more or less clavate form in which the anterior end is narrower than the posterior, and the middle of the ameba is wider than either end . . ." *P. carolinensis* is designated as the type species. The "antlerlike" pseudopods that were considered as one of the important identification marks in Rösels' organism are not mentioned for this genus, although they were used in the characterization of the genus *Metachaos*. Contrary to Schaeffer's statement, the clavate forms of *P. carolinensis* are typically broadest at the anterior extremity (Kudo, 1946) and no "tongues" extending from the ectoplasmic grooves occur in it.

Genus *Flabellula*, according to Schaeffer, is characterized as follows: "During

active locomotion the amebas belonging to this genus have a triangular or fan-shaped body with a broad anterior end. No pseudopods are formed during locomotion, but there may be short blunt scurs in varying number along the anterior edge . . .” Of the four new species he described, three are fan-shaped, but *F. pellucida* is ovoid with its long axis at right angles to the direction of movement, while “a uroid consisting of long, thin, root-like appendages” is said to occur in two species, but to be absent in the other two species.

Time does not permit further examination; suffice it to say that these characterizations of the genera are so ambiguous, indistinct, and lacking sharp demarcation that they are impossible to follow in practice. This is my experience. However, there are some who consider that Schaeffer's genera can be used. Bovee (1954), among others, advocates adoption of Schaeffer's genera and seems to be adding new genera to them. For example, finding an amoeba that showed “morphological characteristics of most amebas placed in the genus *Mayorella* by Schaeffer (1926),” but which produces “one or more tapering, elongate pseudopods which wave actively,” Bovee (1953) created the new genus *Oscillosignum* for it, because he believed that these actively waving pseudopods are generically different from the “determinate, clear pseudopods” of the type species of Schaeffer's *Vexillifera*, which are waved” only preparatory to retraction and never actively as does this amoeba.” Can such a difference in the degree of waving activity of pseudopods be considered a sufficient basis of generic differentiation? To me it cannot be more than a specific peculiarity.

Nucleus. It is universally recognized that the nucleus is the most constant and hence dependable structure in all cells and the least subject to external influence. In the case of parasitic amoebae, generic differentiation based largely upon the nucleus, is clear-cut and used without difficulty. Certain large free-living amoebae possess interphase nuclei that contain many peripheral granules arranged along the nuclear membrane; others show a centrally located prominent endosome with very few if any peripheral granules that may be made a basis for generic differentiation. However, one must remember that interphase nuclei are highly dynamic. Dobell (1943) and Ray and Hayes (1954) observed a form change of the endosome in the nucleus of *Dientamoeba* and *Hartmannella*, respectively. In an amoeba under cultivation, I have found that when this animal is inactive, its nucleus is a spherical body in which a large spherical endosome is suspended in the center. However, when it is moving and feeding, the endosome undergoes an extremely rapid change in form that could best be described as “amoeboid” and, when the organism ceases locomotion and feeding, the structure becomes a centrally located spherical body. Thus far no attempt has been made to differentiate amoebae on the basis of the nuclear structure, but I entertain the hope that the time will come when we can subdivide the genus *Amoeba* by utilizing this character.

The nuclear division patterns that have been used by some investigators for differentiating the genera of small amoebae (Singh, 1952) cannot be applied to large amoebae, since nuclear division is known in a relatively small number of species.

The number of nuclei present in an organism is easily determined and ap-

pears to be constant. Greeff (1874), who discovered *P. palustris*, considered multinucleation the chief characteristic of the organism for which he created the genus. Leidy (1879) maintained a similar view, but Penard (1902) characterized *Pelomyxa* by a slow movement and possession of "symbiotic" bacteria, and he did not attach any significance to the number of nuclei. Schaeffer (1926, 1937) followed Penard and placed both *A. proteus* and *P. carolinensis* in the genus *Chaos*.

The multinuclear characteristic is permanently fixed in all three species of *Pelomyxa*; namely, *P. palustris* (Kudo, 1957), *P. carolinensis* (Wilson, 1900; Kudo, 1946, 1947, 1949), and *P. illinoisensis* (Kudo, 1951; McClellan, 1954). All multiply by a simple or multiple plasmotomy and give rise to multinucleate progeny. Recent observation by Prescott (1956), who obtained two clones of *P. carolinensis* from two artificially produced uninucleate fragments, demonstrates beyond doubt that the multinucleation in this organism is a constant and permanent characteristic.

Uninucleate amoebae divide by binary fission and give rise normally to two smaller uninucleate individuals. Mingled among the uninucleate amoebae, a certain number of animals containing more than one nucleus have been observed by many workers. Stolc (1905, 1906) found *A. proteus* with two, three, and four nuclei, which eventually divided into uninucleates. He considered that aging of the culture, lack or excess of nutriment, or unfavorable season may have been responsible for the appearance of these supernucleates, although fusion of uninucleates cannot be ruled out. Schaeffer (1916) found binucleate animals in *A. discoides* and remarked that the binucleate condition indicates some derangement within the amoeba that often, though by no means always, terminates fatally.

Doflein (1918) found *A. proteus* with 1 to 8 nuclei. In a series of observations, he found that quadrinucleates divided into 2 binucleates that in turn divided into uninucleates. Levy (1924) also found *A. proteus* with 2, 3, or 4 nuclei in his cultures, and he observed that an increase in the number of nuclei was accompanied by a decrease in the division rate and by an increase in the mortality rate. He was inclined to think that supernucleation occurs in old cultures in which conditions unfavorable to cytoplasmic division develop by the accumulation of the excretion products of the amoebae and coexisting organisms, by a concentration of the organic substances that are left behind as the water evaporates, or by a combination of these changes. Johnson (1930) reported *A. proteus* containing as many as 6 nuclei, which divided by multiple fission into uninucleates. Chalkley (1931) also noted individuals of the same species with 2 to 6 nuclei. These observations seem to point to the conclusion that in *A. proteus* more than one nucleus may occur when the division of the cytoplasm does not follow that of the nucleus.

All reports indicate that individuals containing more than 1 nucleus occur in a small number. For example, Chalkley (1931) took at random some 200 individuals of *A. proteus* from a culture, and found the following percentages for different nucleates: uninucleates, 91.8; binucleates, 5.7; trinucleates, 1.4; and quadrinucleates, 1.1. Based upon published reports and my own observation,

it seems reasonable to assume that supernucleation in normally uninucleate amoebae is an abnormal or a temporary condition and constitutes a small minority. Therefore, in agreement with Greeff (1874), Leidy (1879), Wilson (1900), and others, I maintain that the number of nuclei is permanently fixed, and that it makes a precise and easily determinable criterion for defining the genera *Amoeba* and *Pelomyxa*. It may be added here that this generic criterion has been used in other groups of protozoa. For example, Jepps and Dobell (1918), in creating *Dientamoeba*, emphasized that "each individual is typically binucleate"; and Metcalf (1923) used the number of nuclei and body shape in subdividing *Protociliata* into 2 subfamilies and 4 genera.

Cytoplasmic structure. As mentioned already, Penard (1902) held that the presence of bacteria in *P. palustris* as symbionts (in text) or parasites (in illustration) was the most important characteristic of *Pelomyxa*. The exact relationship between the bacteria and the sarcodinan is still unknown. Short (1946) and Leiner *et al.* (1954) apparently follow Penard and suggest that *P. carolinensis*, which does not contain bacteria, be placed in another genus. Several protozoan genera are now known in which some species contain foreign organisms as symbionts, while other species placed in the same genus do not. For example, *Paramecium bursalia* contains zoochlorellae, while other species of *Paramecium* do not. Should we then remove bursaria from the genus *Paramecium* and place it in another genus? In my opinion, the presence of foreign organisms in *P. palustris* is nothing more than a specific character.

Physiological characteristics. Up to the present time, no one of the numerous amoebae has been cultivated *in vitro* free from other organisms, which explains our poor knowledge on precise physiological activities of the amoebae. Recently, numerous publications are bringing to light certain information concerning biochemical properties of *A. proteus* and *P. carolinensis*. Andresen and Holter (1949) reported the presence of two enzymes in them, as well as in *P. palustris*. However, their suggestion that the difference in amounts of the enzymes may be made a basis for generic differentiation is difficult to comprehend, since, as I have already remarked (Kudo, 1952, 1957), they have not eliminated the possibility that the results may indicate the physiological differences of the specimens used for the observation, rather than the "generic" differences.

While most of us who are engaged in the study of amoebae seem to agree that the genus *Amoeba* includes a great variety of amoeboid organisms and should be subdivided into a number of genera, there is at present no clear-cut criterion, agreeable to all concerned, on which genera can be founded. Let us hope that more precise information on morphologic and physiological characters of the amoebae in relation to environment will be forthcoming, which will lead to the establishment of distinctly defined genera in place of the genus *Amoeba*. In the meantime, this genus must be retained. I must regretfully agree with Penard who, about 60 years ago, felt the need of subdividing the genus *Amoeba*, but thought that the time was not yet ripe for it.

Summary

Chaos chaos Linnaeus and *Proteus diffluens* Müller cannot be identified with any of the known amoebae. *Pelomyxa carolinensis* Wilson is the valid name

for the organism described and named by Wilson (1900). *Amoeba proteus* (Leidy, 1878) Schaeffer, 1916, is the valid name of the common amoeba. Generic characterizations based upon external morphology as conceived by Schaeffer are indistinct and confusing, and are difficult to follow in practice. Retaining the genus *Amoeba* is advocated.

References

- ANDRESEN, N. & H. HOLTER. 1949. The genera of amoebae. *Science*. **110**: 114-115.
- BLOCHMANN, F. 1894. Kleine Mitteilungen über Protozoen. *Biol. Zentr.* **14**: 82-91.
- BOVEE, E. C. 1953. *Oscillosignum* nov. gen. *proboscidium* nov. sp., type form of its genus, Family Mayorellidae, Order Amoebida. *Trans. Am. Microscop. Soc.* **72**: 328-332.
- BOVEE, E. C. 1954. Morphological identification of free-living Amoebida. *Iowa Acad. Sci.* **60**: 599-615.
- CHALKLEY, H. W. 1931. The chemistry of cell division. II. The relation between cell growth and division in *Amoeba proteus*. U. S. Publ. Health Repts. **46**: 1736-1754.
- DOBELL, C. 1943. Researches on the intestinal protozoa of monkeys and man. XI. *Parasitology*. **35**: 134-158.
- DOPLEIN, F. 1918. Die vegetative Fortpflanzung von *Amoeba proteus* Pall. *Zool. Anz.* **49**: 257-268.
- FROMENTEL, E. DE. 1874. Étude sur les microzoaires ou infusoires proprement dits. Paris, France.
- GREEFF, R. 1874. *Pelomyxa palustris* (Pelobius), ein amoebenartiger Organismus des süßen Wassers. *Arch. mikroskop. Anat.* **10**: 53-73.
- JEPPI, M. M. & C. DOBELL. 1918. *Dientamoeba fragilis* n.g., n.sp., a new intestinal amoeba from man. *Parasitology*. **10**: 352-367.
- JOHNSON, P. L. 1930. Reproduction in *Amoeba proteus*. *Arch. Protistenk.* **71**: 463-498.
- KEPNER, W. A. & J. G. EDWARDS. 1917. Food-reaction of *Pelomyxa carolinensis*. *J. Exptl. Zool.* **24**: 381-407.
- KUDO, R. R. 1946. *Pelomyxa carolinensis* Wilson. I. General observation on the Illinois stock. *J. Morphol.* **78**: 317-351.
- KUDO, R. R. 1947. *Pelomyxa carolinensis* Wilson. II. Nuclear division and plasmatomy. *J. Morphol.* **80**: 93-144.
- KUDO, R. R. 1949. *Pelomyxa carolinensis* Wilson. III. Further observations on plasmatomy. *J. Morphol.* **85**: 163-176.
- KUDO, R. R. 1951. Observations on *Pelomyxa illinoisensis*. *J. Morphol.* **88**: 145-184.
- KUDO, R. R. 1952. The genus *Pelomyxa*. *Trans. Am. Microscop. Soc.* **71**: 108-113.
- KUDO, R. R. 1957. *Pelomyxa palustris* Greeff. I. Cultivation and general observations. *J. Protozool.* **4**: 154-164.
- LEIDY, J. 1875. Remarks on rhizopods. *Proc. Acad. Natl. Sci. Philadelphia.* : 415.
- LEIDY, J. 1878. *Amoeba proteus*. *Am. Naturalist.* **12**: 235-238.
- LEIDY, J. 1879. Freshwater rhizopods of North America. *Rept. U. S. Geol. Survey Terr.* **12**: 1-324.
- LEINER, M. 1924. Das Glykogen in *Pelomyxa palustris* Greeff mit Beiträge zur Kenntnis des Tieres. *Arch. Protistenk.* **47**: 253-307.
- LEINER, M., M. Wohlfeil & D. Schmidt. 1954. *Pelomyxa palustris* Greeff. *Ann. sci. nat. Zool.* **16**: 537-594.
- LEVY, J. 1924. Studies on reproduction in *Amoeba proteus*. *Genetics.* **9**: 124-150.
- LINNAEUS, K. 1758. *Systema Naturae*. 10th ed. Brit. Museum, Nat. Hist. London, England.
- LINNAEUS, K. 1767. *Systema Naturae*. 12th ed.
- MAST, S. O. 1938. *Amoeba* and *Pelomyxa* vs. *Chaos*. *Turtlox News.* **16**: 56-57.
- MAST, S. O. & P. L. JOHNSON. 1931. Concerning the scientific name of the common large amoeba, usually designated *Amoeba proteus* (Leidy). *Arch. Protistenk.* **75**: 14-30.
- MCCLELLAN, J. F. 1954. Observations on nuclear division in *Pelomyxa illinoisensis* Kudo. *Doct. Thesis. Univ. Ill. Urbana, Ill.*
- MCGUIRE, I. P. 1932. *Pelomyxa carolinensis* in Great Britain. *Nature.* **130**: 168-169.
- METCALF, M. M. 1923. The opalinid ciliate infusorians. *Bull. Smithsonian Inst. U. S. Natl. Museum.* **120**: 1-484.
- MÜLLER, O. F. 1786. *Animalcula Infusoria Fluvialia et Marina.*
- PENARD, E. 1902. Faune rhizopodique du Bassin du Léman. Geneva, Switzerland.
- PRESCOTT, D. M. 1956. Mass and clone culturing of *Amoeba proteus* and *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **30**: 1-12.

- RAY, D. L. & R. E. HAYES. 1954. *Hartmannella astronyxis*: a new species of free-living ameba. J. Morphol. **95**: 159-188.
- RICE, N. E. 1945. *Pelomyxa carolinensis* (Wilson) or *Chaos chaos* (Linnaeus)? Biol. Bull. **88**: 139-143.
- RÖSEL VON ROSENHOF, A. J. 1755. Der kleine *Proteus*. Monatlichherausgegebenen Insecten-Belustigung. **3**: 622-624.
- SCHAEFFER, A. A. 1916. Notes on the specific and other characters of *Amoeba proteus* Pallas (Leidy), *A. discoides* spec. nov., and *A. dubia* spec. nov. Arch. Protistenk. **37**: 204-228.
- SCHAEFFER, A. A. 1926. Taxonomy of the amebas. Papers Dept. Marine Biol. Carnegie Inst. Washington. **24**: 1-116.
- SCHAEFFER, A. A. 1937. Morphology, behavior and reproduction in Type A and Type B of *Chaos chaos* Linnaeus, the giant multinuclear ameba of Rösel. Biol. Bull. **73**: 355.
- SCHAEFFER, A. A. 1937a. Rediscovery of the giant ameba of Rösel, *Chaos chaos* Linnaeus, 1767. Turtox News. **15**: 114.
- SHORT, R. B. 1946. Observations on the giant amoeba *Amoeba carolinensis*. Biol. Bull. **90**: 8-18.
- SINGH, B. N. 1952. Nuclear division in nine species of small free-living amoebae and its bearing on the classification of the order Amoebida. Phil. Trans. Roy. Soc. London. **B236**: 405-461.
- STILES, C. W. 1905. Public health papers and reports. Am. Public Health Assoc. **30**: 293.
- STILES, C. W. & W. C. BOECK. 1923. The nomenclatorial status of certain protozoa parasitic in man. Bull. Hyg. Lab. U. S. Public Health Service. **133**: 92-188.
- STOLC, A. 1900. Beobachtungen und Versuche über die Verdauung und Bildung der Kohlenhydrate in einen amoebenartigen Organismus, *Pelomyxa palustris* Greeff. Z. wiss. Zool. **68**: 625-668.
- STOLC, A. 1905. Über die Teilung des Protoplasmas in mehrkernigen Zustände. Nach den Untersuchungen an mehrkernigen Formen der *Amoeba proteus*. Arch. Entwicklungsmech. Organ. **19**: 631-647.
- STOLC, A. 1906. Plasmodiogonie, eine Vermehrungsart der niedersten Protozoen. Nach den Untersuchungen an mehrkernigen Formen der *Amoeba proteus*. Arch. Entwicklungsmech. Organ. **21**: 111-125.
- WILSON, H. V. 1900. Notes on a species of *Pelomyxa*. Am. Naturalist. **34**: 535-550.

Part II. Physical Studies and Cell Division

SOL-GEL TRANSFORMATIONS IN AMOEBAE

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It is a well-established fact that all living cells, at some stage in their physiological activity, perform work as a result of the utilization of mechanical energy. This may be exhibited as cytokinesis, karyokinesis, locomotion, or a host of other functional processes. The amoeba, the organism of prime concern here, presents us with the opportunity to study, at a single cell level, the mechanisms involved in the constant expenditure of mechanical energy during the process of locomotion. The object of this paper is to review and perhaps add somewhat to our knowledge of amoeboid locomotion and, in doing so, to emphasize the conversion of metabolically derived chemical energy into mechanical energy and ultimately into mechanical work.

Over a period of years many investigators have expressed the belief that all mechanical energy produced by any living cell has as its basis a contractile mechanism. Certainly, the isolation of a myosinlike contractile protein in the slime mold by Ts'o *et al.* (1956) and a similar isolation in sarcoma cells by Hoffman-Berling (1956), along with the earlier work of Mirsky (1936) on *Arbacia* eggs and Loewy (1952), also on slime mold, lend credence to such a belief. Additional evidence is also presented by the work of Hoffman-Berling (1954*a, b, c*) in which he reports the existence of a contractile phenomenon dependent on adenosine triphosphate (ATP) in the anaphase movement of chromosomes and in the deepening of division furrows in glycerinated cell models. Recently Mitchison (1952) has proposed an "expansion" theory of cytokinesis, but even this theory recognizes a contractile phase. Mitchison's data have been adequately analyzed on the basis of a contractile mechanism by Marsland and Landau (1954) and need not be discussed further here.

In proposing a contractile mechanism for amoeboid locomotion one must assume a macromolecular structure of some type. Frey-Wyssling (1953) has thoroughly presented the necessity for such an assumption. This structure must have the ability to contract and must be highly labile. Its lability is a prerequisite for the conservation of the structural elements, that is, the structure must be constantly formed, utilized, and then disrupted in a cyclical manner, all as a result of rather minute changes in the cytoplasmic environment. This, of course, indicates a dynamic equilibrium between a less highly organized state and a more highly organized structural state. The former is commonly referred to as the *sol* or fluid state, while the latter is termed the *gel* or structural state. The equilibrium properties of such "biogels" and their ability to perform work are thoroughly discussed and documented in the work of Katchalsky (1954). General agreement with the belief that the gel structure is that of a three-dimensional protein network has been pointed out in a review by

Ferry (1948). It is the *formation* of this structure that remains a subject of some controversy. The contractile ability of the gel would also seem to be a point of agreement, while the nature of the *conversion* of chemical to mechanical energy is greatly disputed. Each of these intriguing facets of the problem will be discussed later in this paper.

Several early workers in the field recognized the gel layer in the amoeba and ascribed to it a role in locomotion. Montgomery (1881), Rhumbler (1905), Gruber (1912), and Hyman (1917) all developed theories involving the utilization of a contracting gel and expanding sol. However, it remained for Mast (1926 and 1931), in his classic analysis of the microscopic structure of the amoeba, to formulate a theory of gel-contractility involving a dynamic sol-gel equilibrium. It is this theory that will be discussed later in the light of recent experiments.

Characterization of the Plasmagel Layer

Marsland and Brown (1936) and Brown and Marsland (1936) reported the effect of high hydrostatic pressures on *Amoeba dubia* and *Amoeba proteus*. The application of these pressures produced a cessation of locomotion and eventual collapse of pseudopodia. Significantly, centrifugal studies of similarly pressurized specimens indicated a marked decrease in the "relative viscosity" of the plasmagel layer. These observations were difficult to interpret until Freundlich (1937) analyzed gel formation. Freundlich concluded that there were basically three types of gel formation: (1) gelation accompanied by a loss in volume and heat, and exemplified by gelatin; (2) gelation accompanied by an increase in volume and absorption of heat as exemplified by methyl cellulose; and (3) gelation accompanied by neither heat nor volume change, as exhibited by sodium oleate. It seemed evident from this analysis, then, that application of high hydrostatic pressure could prevent the gelation of methyl cellulose by opposing the volume increment ($+\Delta V$), and assist in the formation of a gelatin gel by aiding the loss of volume ($-\Delta V$). Marsland and Brown (1942) subjected gelatin and methyl cellulose to high pressures and found that a gelatin sol would exhibit graded increments of relative viscosity upon stepwise increase of pressure levels, and revert back to the sol state upon return to atmospheric pressure. Methyl cellulose gels behaved in an exactly opposite fashion, decreasing in "viscosity" with increased pressure and returning to the gel state upon release of pressure. These experiments not only added confirmatory evidence to Freundlich's analysis, but also further established the sol-gel transformation as a reversible equilibrium process. In addition, Marsland and Brown investigated the properties of an actomyosin gel and found that this conformed to the findings on methyl cellulose, with one notable difference. An equilibrium state with regard to viscosity level of the myosin gel was attained very rapidly upon compression (within 2 sec.). After decompression, only 5 to 10 min. were needed for complete regelation. On the other hand, the methyl cellulose gel required at least 2 hours in either direction. From these data one may assume, then, that paralleling methyl cellulose, a protoplasmic gel, or "biogel" such as myosin, absorbs heat or energy and displays a $+\Delta V$ upon gela-

tion, releases energy and displays a $-\Delta V$ upon solation and, in addition, is geared for extremely rapid transformations in either direction. On this basis, manipulation of environmental temperatures and application of hydrostatic pressures should serve as useful physical methods for analyzing the sol-gel transformation within the cell.

Accordingly, Landau *et al.* (1954) investigated the plasmagel of *A. proteus* and *A. dubia* by means of both temperature and pressure manipulations. Their observations will be discussed in some detail.

Effects on form and locomotion. Experiments performed at 25° C. confirmed the earlier findings of Marsland and Brown (1936). These show that the plasmagel is more readily solated in newly formed pseudopodia, and support the view proposed by Mast (1926) that a gradient exists in the rigidity of the plasmagel tube, decreasing as one proceeds from the base to the tip of the pseudopodium. At this temperature, low pressure levels (2000 psi) cause a disappearance of the hyaline cap at the tip of a pseudopodium, higher levels (3000 to 4000 psi) stop all movement and cause the formation of bulbous pseudopodial tips, and still higher levels (6000 psi) cause a complete breakup into spheres. This general pattern of solation caused by relatively high pressure levels is presented in FIGURE 1.

Influence of temperature. Similar experiments, performed at 20°, 15°, and 10° C., gave a series of parallel results. In general, comparable changes in form occurred at each temperature level. However, the time necessary to reach the ultimate spherical shape increased with each decrease in temperature, the maximum time being 15 to 20 min. at 10° C. At 10° C. the breaking up into several spheres rarely occurred, the resultant form being one large sphere. However, of greatest significance was the observation that the pressure level required to

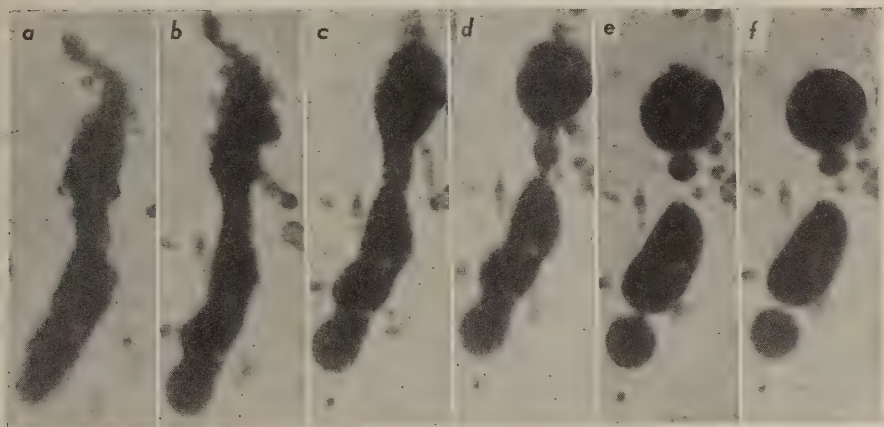


FIGURE 1. Collapsing and "pinching off" of pseudopodium, which occurs when the amoeba is suddenly exposed to pressures above a critical level. In this case, the pressure was 7000 psi at 25° C. (a) One minute before compression; (b) five seconds after compression; (c) one minute later; (d to f) successive photos taken at 1.5-min. intervals, thereafter pressure maintained. Reproduced by permission from Marsland, 1956.

produce each change became progressively lower as the temperature was reduced.

It is easily recognized that complete solution of the gel structure converts the amoeba into what is essentially a fluid cylinder of nonmiscible liquid suspended in water and subject to the tensional forces of the surface in the system. The reorganization of a fluid cylinder into drops will occur when the length exceeds π times the diameter, provided the viscous (or plastic) resistance to deformation is not too great. This explains the time lag and resistance to breaking up at lower temperatures for, while the gel strength may decrease with decreasing temperature, the viscosity of the sol probably increases. It also affords a criterion for quantitative temperature-pressure measurements on a morphologic basis. The critical pressure level at any given temperature (the pressure that would cause complete solution) was designated as the minimum pressure that would result in the attainment of a spherical shape within 20 min. by 75 per cent of the specimens. The results of these experiments are shown in FIGURE 2, the critical end point in FIGURE 3a. It is to be noted that the critical pressure decreased by 1000 psi for each 5° C. decrease in temperature. Centrifugal studies were also performed and they showed quite conclusively that plasmagel

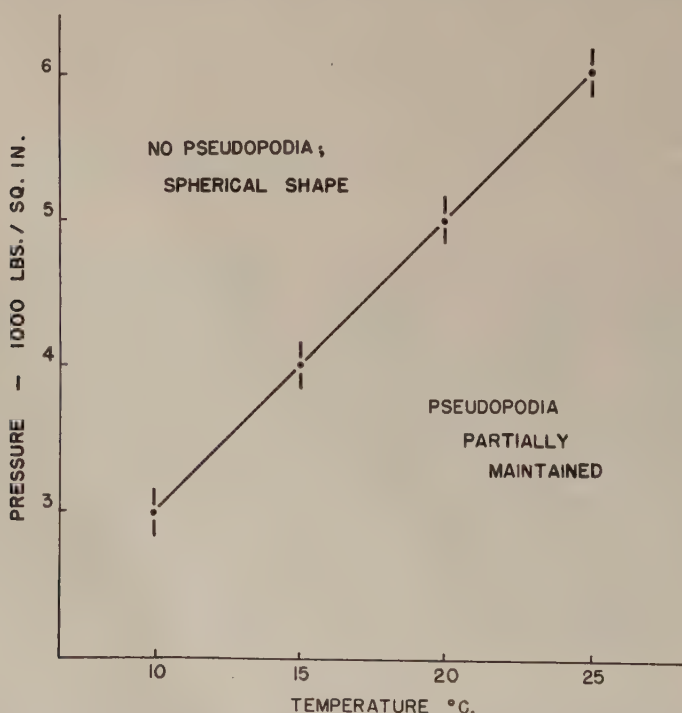


FIGURE 2. Relations of pressure and temperature to the capacity of *Amoeba proteus* to form and maintain pseudopodia. Note that the critical pressure above which the cells cannot form or maintain any pseudopodia is increased by 1000 psi with each temperature increment of 5° C. Reproduced by permission from Landau *et al.*, 1954.

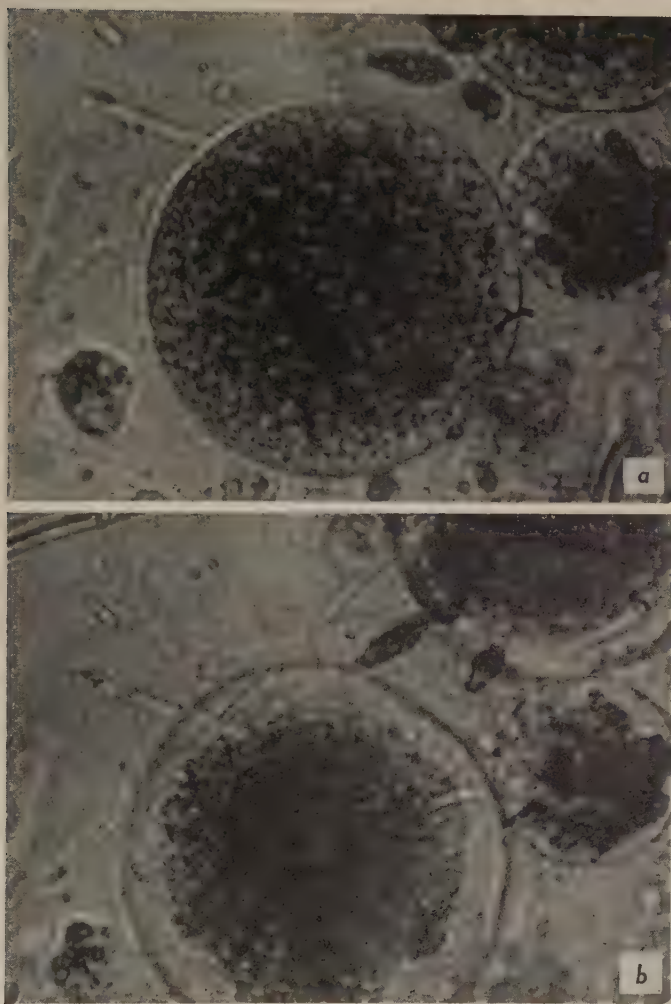


FIGURE 3. (a) *Amoeba proteus* after 20-min. exposure to a pressure of 6000 psi at 25° C. Note the completely spherical shape and the pinched-off sphere at the right. (b) The same amoeba 15 sec. after decompression. Note the contracted central mass and broad hyaline zone.

rigidity did, indeed, vary in accordance with the data arrived at by use of an over-all morphologic criterion. The plasmagel layer has numerous granules entrapped within its network. The time necessary to clear these granules from the hyaline zone (of the centrifuged specimen) at constant force, under given conditions of pressure and temperature, was adopted as a measure of gel strength. The results are shown in FIGURE 4.

Decompression and reversibility. Perhaps the most important of the observa-

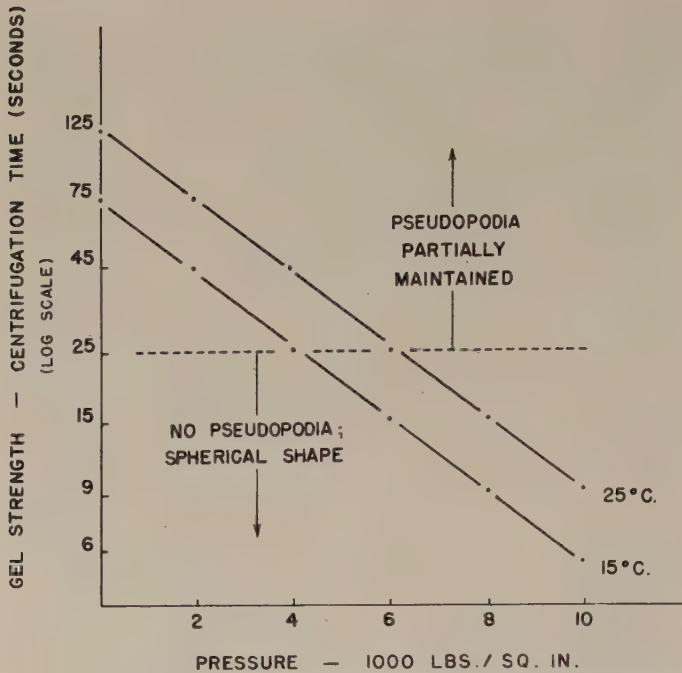


FIGURE 4. Structural strength of plasmagel of *Amoeba proteus* plotted as a function of pressure at 15° and 25° C. Note that the two curves are parallel and that the critical gel strength, below which no pseudopodia can be maintained, is the same at both temperatures. Reproduced by permission from Landau *et al.*, 1954.

tions made during these experiments was recorded immediately following decompression. Upon release of pressure, the specimen maintained its spherical shape and exhibited no change for perhaps 12 to 15 sec. At this point the granular cytoplasm pulled away from the cell membrane, exhibiting a massive generalized contraction. The specimen now consisted of a centralized contracted mass (about 50 per cent of the total volume), a broad, clear fluid zone, and the membrane. This condition is shown in FIGURE 3*b*. Following this by several (10 to 20) sec., the contracted mass began to disperse and the granules moved into the fluid zone. At this point the membrane began to bulge conspicuously, and soon pseudopodial activity returned. Within 20 to 30 min., the amoeba could not be distinguished from the normal precompression specimen.

Several important conclusions have been reached as a result of these experiments: (1) the maintenance of the plasmagel at a specific level of structural strength or rigidity is a prime requisite for the maintenance of form and locomotion in the amoeba; (2) sol-gel transformation in the amoeba is a reversible equilibrium phenomenon; (3) the formation of the gel is an endothermic, energy-absorbing process characterized by a positive volume increment; and (4) the gel possesses a rather high degree of potential contractility.

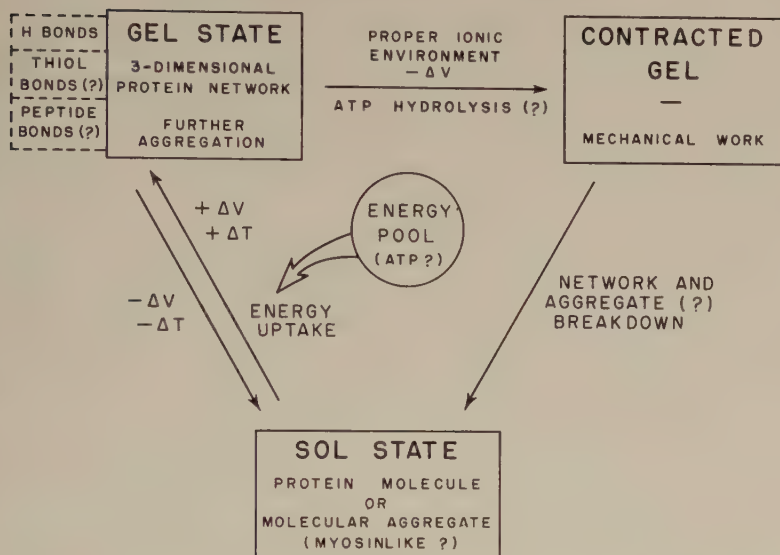


FIGURE 5. A schematic representation of the sol-gel phenomenon. Note that the sol-to-gel stage is reversible. The hydrolysis of ATP is arbitrarily placed between the gel and the contracted gel. This may actually occur at any stage (see text). Ionic "salt" linkages, perhaps the most important in polymer and network formation, are not represented on this chart.

The Dynamic Equilibrium

A schematic representation of the cyclical sol-gel transformation phenomenon is presented in FIGURE 5. It is to be noted that the gel formation phase is an equilibrium or reversible stage and that the transformation to a contracted gel and, following that, the return to the sol state are represented as irreversible. The present status of experimental evidence on the molecular rearrangement involved in gel formation and contraction is such that no definitive theory may be accepted as having been conclusively proved.

In the scheme presented in FIGURE 5 it is considered, somewhat arbitrarily, that the potential energy of the ATP molecule is expended in the process of contraction. This is broadly conceived as the "hydrolysis" of ATP, although transfer of the phosphate bond energy through an intermediate compound and a subsequent hydrolysis is certainly possible, if not highly probable. This energy utilization may actually occur, on the basis of varied interpretation, either in the gel formation, contraction, or break-down stage or, possibly, in all three. The object here is to present all three aspects and suggest experiments that may perhaps eliminate some of the controversy.

Network formation. The massive unfolding or denaturing of globular proteins to form fibers as proposed by Goldacre and Lorch (1950) seems unlikely. Similarly, the coiled chain of globular protein presented by Kopac (1951) seems an unnecessary step in the formation of a network. The association of native, macromolecular monomers into polymeric chains as shown by Waugh (1957),

along with the extensive cross-linking as proposed by Anderson (1956a, 1956b), would present a structural complex exhibiting a yield value (as a true gel). Such a structure would also exhibit the lability or reversibility experimentally shown to exist in living cells. Gross (1957) points out that the bonds available for linkages in intact globular protein molecules and nucleoprotein are identical with those available in extended polypeptides, thereby making any extensive unfolding unnecessary. Chief among these would be, perhaps, H bonds and ionic or "salt" linkages—both show a large positive ΔV of formation and would, of course, be prevented or reversed by high hydrostatic pressures. Anderson (1956a) considers that globular protein molecules may be bound to one another to form polymeric chains by organic polycations (Gross, 1957, for role of inorganic polycations) with extensive cross linkages being formed by small amounts of ribonucleic acid (RNA) or acid polysaccharides.

The unbound protein of the sol state is represented in FIGURE 5 as a myosin-like protein probably existing in an aggregate form. The work of Ts'o *et al.* (1956) in isolating what they have called myxomyosin from a slime mold is the basis for beginning the schematic representation of FIGURE 5 with a myosinlike molecule. The evidence presented by Parrish and Mommaerts (1954) and Laki and Carroll (1955) shows that myosin reversibly aggregates from 0 to 25° C. and that at 25° C. it probably exists in the "dimer" form.

If one is to assume that ATP is "split" during gel formation, the potential energy of the phosphate bond may be thought of as being involved in the exposure of the intermolecular bonding sites on a myosinlike molecule. However, high pressure has been shown by Laidler and Beardall (1955) to enhance the hydrolysis of ATP by myosin ATPase, provided enzyme-substrate binding has already occurred. Therefore, in this instance pressure would have to act in two opposing capacities: (1) initially aiding in the exposure of bonding sites; and (2) at the same time preventing the formation of such bonds. Perhaps it is somewhat more feasible to consider that the entire ATP moiety is merely bound to the protein molecule during the process of gel formation. By neutralizing certain positive charges, the ATP would then afford the molecule a greater symmetry with respect to negative charges. These symmetrically located charges would then be available for intermolecular polycationic binding. Such ATP adsorption would display a positive ΔV and establish the network as a source of high potential energy.

Landau *et al.* (1955) and Zimmerman *et al.* (1958) have shown that addition of small amounts of ATP to the environmental medium does, in fact, produce increased cortical gel strength in both marine eggs and amoebae. Whether this is due to its splitting or solely to its binding is not known. It has also been reported by Zimmerman *et al.* (1957) that sulfhydryl reagents will decrease the cortical gel strength of marine eggs. This last finding conforms with either of the previous hypotheses of gel formation for, by preferentially binding the ATPase sites known to exist on the myosin molecule, such sulfhydryl reagents may block ATP hydrolysis, whereas, by their over-all effect on the metabolism of the cell, they may decrease the amount of ATP available for binding. Weber (1955) has reported that salyrgan, a potent sulfhydryl reagent, does not block the binding of ATP in glycerinated models. When ATP is added to a salyr-

gan-blocked system, the result is a "plasticizing" or "relaxing" effect presumably due to the binding of the entire ATP moiety. This "plasticizing" process is reversible and may be considered under these conditions as a re-extension of the gel network to its precontraction state. Mommaerts (1956) has suggested the possibility of a one-site enzyme-substrate binding as a "plasticizing" requirement with a two-site binding as a "contractile" or "hydrolytic" requirement.

Gel contraction. First, we shall start with the concept of ATP hydrolysis during gel *formation*. In accordance with Anderson's (1956a) theory, such action would decrease the number of polyanionic charges, thus favoring gelation. Continuing hydrolysis would produce a further shift in favor of polycations and result in a contraction. Finally, a shift in favor of polyanions would cause a return of the system to a discrete molecular state. All the polyelectrolyte shifts are presumably based upon changes in their rates of synthesis or break down; therefore this final step (polyanion increase) possibly might be the stimulated production of ATP.

The remaining theories to be discussed here are based on the *binding* of ATP to the protein molecule during network *formation* and necessitate the assumption of a masking factor to prevent any extensive ATPase activity until the time of contraction.

Weber (1957) and Szent-Györgyi (1957) believe that it is the "splitting" of ATP that causes contraction, while Morales *et al.* (1955) and Flory (1957) attribute the contraction to "binding" of ATP, albeit by different mechanisms.

Flory (1957) proposes a contraction as a result of the binding of ATP on the basis of a dimensional and structural change of the myosin from the crystalline to the amorphous state. This is assumed to be analogous to the melting of a simple crystalline substance. As ATP is bound to the protein molecule, this "complex" can no longer be accommodated in the crystalline state, and a shift to the amorphous polymeric state occurs. An increase in the concentration of ATP (the "complexing" reagent) will shift the crystalline-amorphous equilibrium in the direction of an increase in "melting." Contraction would of necessity accompany such a "melting" equilibrium shift.

Morales *et al.* (1955) believe that electrostatic forces constitute the basis for contraction. The following is perhaps an oversimplified description of this theory: (1) the myosin molecule is predominantly negatively charged; (2) certain polycations (Mg^{++} , Ca^{++} ?) are adsorbed to the myosin molecule, yielding a protein-cation complex now predominantly positively charged; (3) these positively charged sites act to repel each other and maintain the molecule in an extended condition; (4) the polyanion ATP is bound to the molecule, effectively neutralizing certain of these positive charges; and (5) when sufficient ATP is bound, these repulsive forces are effectively eliminated and the molecule contracts.

Weber (1957) and Szent-Györgyi (1957) are, perhaps, the chief proponents of the "ATP-splitting" theory of muscular contractions. The hydrolysis of ATP is presented as being the prime factor in the production of the contracted state. This theory basically proposes that the energy produced by ATP hydrolysis is transferred, in a rather complex fashion, between subunits of the

myosin molecule, and is thereby utilized directly for the molecular reorientation resulting in contraction.

In all theories it would seem that the "triggering" mechanism for contraction lies in the achievement of a specific level of ATP concentration along with a properly balanced ionic milieu.

It is not within the scope of this paper to analyze critically any of the foregoing contraction theories; suffice it to say that there exists a myriad of experimental evidence that, depending upon personal interpretation, may lend support to any or all theories. However, there are several observations made during pressure experiments on amoebae and dividing eggs that may indicate support for the ATP-splitting theory.

If ATP is hydrolyzed during contraction and is the basis for contraction, then application of high hydrostatic pressures should initially assist this phase by virtue of its reinforcing the negative ΔV of hydrolysis. This abetting of contractility seems to occur. Application of high pressure results in the following immediate effects: (1) the furrow of a dividing frog's egg deepens momentarily (Marsland and Jaffee, 1949); (2) cleavage-furrow formation in marine eggs proceeds at a more rapid pace; (3) an amoeba will at times extend a short, slender pseudopodium; and (4) an amoeba subjected to an ATP concentration that will cause over-all contraction and the presence of large vacuoles (Kriszat, 1949) will produce small, bleblike pseudopodia that are practically devoid of granules.

All of these events are momentary, and they occur almost instantaneously upon application of pressure, immediately subsequent to which the typical effects of solation become apparent. In the egg, the cleavage furrow recedes and, in the amoeba, the previously described changes occur. In each instance, the contractile process has already begun at the time of pressure application.

Network breakdown. If one accepts the "binding" hypothesis, then ATP hydrolysis would be assumed to occur following contraction, and the resulting negative ΔV and change in structural configuration of the molecule (Laidler and Beardall, 1955) might tend to disrupt linkages and cause network and polymer breakdown. If one accepts the "splitting" hypothesis, then breakdown will occur as a consequence of contraction.

Proposed experiments. The events involved in decompression afford what would seem to be an ideal situation for the stepwise analysis of a complete cyclical change in the sol-gel phenomenon. FIGURE 3a represents the contraction stage and, undoubtedly, the 10- to 15-sec. interim represents the formation of the gel. Following contraction, there exists another 10- to 15-sec. interval involved in network break down.

Fleckenstein *et al.* (1954) and Mommaerts (1954) have reported that they can find no ATP breakdown at the time of muscle contraction. The application of their methods to the above-mentioned changes in the amoeba may produce additional significant data with respect to the over-all energetics of the system.

Mazia and Dan (1952) have designed a method for the isolation of the spindle apparatus in dividing cells. The application of this or similar methods to the

amoeba at the stage of full contraction may result in the isolation of the contractile substance as a structural entity and permit subsequent analysis.

Amoeboid Locomotion

The results of the pressure-temperature experiments lead to substantial agreement with the "contractile theory of amoeboid locomotion" as proposed by Mast (1926). Forward streaming of the fluid protoplasm, or plasmasol, is caused by a contractile force exerted by the plasmagel at the posterior portions of the amoeba. New gel is being constantly formed at the sides of the advancing pseudopodium, affording it with structural reinforcement as it lengthens. In turn, the contracted gel undergoes solation, supplying the plasmasol with the elemental molecular components for re-gelation at the anterior portions of the cell.

In postulating this type of mechanism one must attribute a spatial gradient of contractile activity to the plasmagel at the posterior of the cell. The gel would contract and subsequently solate only in the region bordering the plasmasol. As this inner zone solated, a new region of gel would be exposed and then undergo the same sequence of events. Such a gradient contractile activity would allow the noncontracting region to remain in close proximity to the plasmalemma at all times and direct the resultant force toward the plasmasol. The generalized massive contraction and detachment from the membrane as shown in FIGURE 3*b* may then be attributed to the temporary loss of any organized, spatially oriented activity. This loss could reasonably be attributed to the random redistribution of cytoplasmic constituents during the preceding stage of complete solation.

Considering that the plasmagel is constantly exerting a force on the plasmasol, pseudopodial flow should follow the path of least resistance. In fact, the advancing tip of a pseudopodium has been shown to be the point of weakest gelation. Should a weaker site occur, a new direction of flow would ensue. Further evidence along these lines is reviewed by Goldacre (1952). When the cell membrane or plasmalemma at the advancing tip of a pseudopodium is touched by a microneedle, no change in flow direction occurs. However, if the needle brings the membrane in contact with the plasmagel, the direction of flow changes, generally proceeding at an angle to the original path. This may be interpreted as a localized gelation due to membrane-plasmagel contact, which blocks the original path of flow and directs it toward a site of weaker gelation. On the other hand, if the needle passes through the thin gel layer at the tip of the pseudopodium, effectively disrupting it, the plasmasol bursts through the gap and blows out a pseudopodium at the point of penetration.

The plasmalemma, or plasma membrane, undoubtedly has some role in amoeboid movement. In actual forward movement the plasmalemma must maintain points of adhesion to the substratum and must be pulled forward to some extent, in relation to these points, by pseudopodial flow. The spherical shape shown in FIGURE 3 represents a minimum surface-area condition, and this in turn entails a minimum amount of plasmalemma. Any change in shape from the sphere, unless it is accompanied by a decrease in protoplasmic volume

(which does not seem feasible), necessitates an increase in surface area. Such an increase in surface area probably entails the manufacture of additional plasma membrane, whereas decreases would involve reabsorption by the cytoplasm. A possible mechanism for such membrane formation on the basis of the precipitation of soluble molecular complexes has been proposed by Anderson (1957). A comparative electron photomicrographic study involving the plasmalemma of the amoebae in its completely spherical shape (FIGURE 3*b*) and its subsequent pseudopodial condition is presently being undertaken.

Recent experiments by Hirshfield *et al.* (1958) have pointed out that the nucleus plays a role of major importance in sol-gel transformations. Anucleate halves of amoebae show a pronounced decrease in gel strength when compared to their nucleate halves. This is probably the reason for the absence of pseudopodial activity in the pinched-off spheres that are obtained upon application of high pressures. Each pinched-off sphere, regardless of size, undergoes a contraction upon decompression, identical with that of the large main body. This indicates that the molecular elements necessary for contraction are distributed fairly evenly throughout the cytoplasm. However, it is only the main sphere, consisting of the bulk of the cytoplasm and containing the nucleus, that will eventually return to normal pseudopodial activity. Evidently, some elements necessary for maintenance of an uninterrupted cyclical series of events are absent from the smaller fragments.

The role of the nucleus in these events is probably multifold. Mazia and Hirshfield (1950) have shown conclusively that removal of the nucleus greatly upsets phosphate metabolism. It is also possible that manufacture or extension of membrane surface cannot occur in the absence of a nucleus. The governing of such a process by a nuclear "X-substance" has been postulated by Swann (1952). The normal synthesis of RNA, which is probably involved in the cross-linkages of network formation, may also be interrupted by the removal of the nucleus.

Summary

The existence of a dynamic equilibrium between a sol, or fluid state, and a gel, or more highly organized structural state, within the cytoplasm of the amoeba provides the organism with what is probably the most elemental or basic means for the conversion of chemical energy into mechanical work. It is the inherent contractility of this gel state that provides the mechanical force necessary for locomotion, division, excretion, and all other functions of the living organism that are based upon the motility of the cytoplasm.

Experiments have been performed by use of high hydrostatic pressures and variable temperature increments to delineate the nature of the sol-gel equilibrium. It has been shown that this equilibrium may be shifted to the sol state by increasing pressure and decreasing temperature levels and to the gel state by reversing these factors. These findings emphasize the fact that the formation and maintenance of the gel is an energy-requiring process accompanied by a positive volume increment. It has also been shown that the form and movement of the amoeba are based directly upon the formation and maintenance of the gel structure.

A massive contraction of the cytoplasm that follows soon after release of solation pressures delineates the contractile ability of the gel state. The recent findings of myosinlike proteins in amoeboid and other cell forms lend further credence to the idea of a contractile substructure.

A schematic hypothesis of sol-gel transformations as a dynamic equilibrium involving contractility is presented. The application of each of the various theories of muscle contraction to gel contractility is considered in relation to present experimental evidence.

References

- ANDERSON, N. G. 1956a. Cell division I. A theoretical approach to the primeval mechanism, the initiation of cell division, and chromosomal condensation. *Quart. Rev. Biol.* **31**: 169-199.
- ANDERSON, N. G. 1956b. Cell Division II. A theoretical approach to chromosomal movements and the division of the cell. *Ibid*: 243-269.
- ANDERSON, N. G. 1957. Labile colloidal complexes of the cytoplasm. *J. Cellular Comp. Physiol.* **49**: 221-241.
- BROWN, D. E. S. & D. A. MARSLAND. 1936. The viscosity of *Amoeba* at high hydrostatic pressure. *J. Cellular Comp. Physiol.* **8**: 159-165.
- FERRY, J. D. 1948. Protein gels. *Advances in Protein Chem.* **4**: 1-78.
- FLECKENSTEIN, A., J. JANKE, R. E. DAVIES & H. A. KREBS. 1954. Contraction of muscle without fission of adenosine triphosphate or creatine phosphate. *Nature*. **174**: 1081-1088.
- FLORY, P. J. 1957. Crystallinity and dimensional changes in fibrous proteins. *J. Cellular Comp. Physiol. (Suppl.)* **49**: 175-183.
- FREUNDLICH, H. 1937. Some recent work on gels. *J. Phys. Chem.* **41**: 901-910.
- FREY-WYSSLING, A. 1953. *Submicroscopic Morphology of Protoplasm*. Elsevier. New York, N. Y.
- GOLDACRE, R. J. 1952. The action of general anaesthetics on *Amoebae* and the mechanism of the response to touch. *Symp. Soc. Exptl. Biol.* **6**: 128-143.
- GOLDACRE, R. J. & I. J. LORCH. 1950. Folding and unfolding of protein molecules in relation to cytoplasmic streaming, amoeboid movement, and osmotic work. *Nature*. **166**: 497-500.
- GROSS, P. R. 1957. Labile biocolloids, cell division, and the structure of the mitotic apparatus. *Trans. N. Y. Acad. Sci. Ser. II.* **20**: 154-172.
- GRUBER, K. 1912. Biologisch und experimentelle Untersuchungen on *Amoeba proteus*. *Arch. Protistenk.* **25**: 316-376.
- HIRSHFIELD, H. I., A. M. ZIMMERMAN & D. A. MARSLAND. 1959. The nucleus in relation to plasmagel structure in *Amoeba proteus*; a pressure-temperature analysis. *J. Cellular Comp. Physiol.* In press.
- HOFFMAN-BERLING, H. 1954a. Adenosintriphosphat als Betriebsstoff von Zellbewegungen. *Biochim. et Biophys. Acta.* **14**: 182-194.
- HOFFMAN-BERLING, H. 1954b. Die Bedeutung des Adenosintriphosphat für die Zell- und Kernteilungsbewegungen in der Anaphase. *Biochim. et Biophys. Acta.* **15**: 226-236.
- HOFFMAN-BERLING, H. 1954c. Die Glycerin-Wasserextrahierte Telophasezelle als Modell der Zytokinese. *Ibid*: 332-339.
- HOFFMAN-BERLING, H. 1956. Das kontraktile Eiweiss undifferenziertef Zellen. *Biochim. et Biophys. Acta.* **19**: 453-463.
- HYMAN, M. H. 1917. Metabolic gradients in *Amoeba* and their relation to the mechanism of amoeboid movement. *J. Exptl. Zool.* **24**: 381-407.
- KATCHALSKY, A. 1954. Polyelectrolyte gels. *In Progress in Biophysics and Biophysical Chemistry.* **4**: 1-59. J. A. V. Butler & J. T. Randall, Eds. Academic Press. New York, N. Y.
- KOPAC, M. J. 1951. Probable ultrastructures involved in cell division. *Ann. N. Y. Acad. Sci.* **51**(8): 1541-1546.
- KRISZAT, G. 1949. Die Wirkung von Adenosintriphosphat auf Amöben (*Chaos chaos*). *Arkiv Zool.* **1**: 81-86.
- LAIDLER, K. J. & A. J. BEARDALL. 1955. Molecular kinetics of muscle ATP-ase. III. Influence of hydrostatic pressure. *Arch. Biochem. Biophys.* **55**: 138-150.
- LAKI, K. & W. R. CARROLL. 1955. Size of the myosin molecule. *Nature*. **175**: 389-390.
- LANDAU, J. V., A. M. ZIMMERMAN & D. A. MARSLAND. 1954. Temperature-pressure

- experiments on *Amoeba proteus*; plasmagel structure in relation to form and movement. J. Cellular Comp. Physiol. **44**: 211-232.
- LANDAU, J. V., D. A. MARSLAND & A. M. ZIMMERMAN. 1955. The energetics of cell division: effects of adenosine triphosphate and related substances on the furrowing capacity of marine eggs (*Arbacia* and *Chaetopterus*). J. Cellular Comp. Physiol. **45**: 309-329.
- LOEWY, A. G. 1952. An actomyosin-like substance from the plasmodium of a *Myxomycete*. J. Cellular Comp. Physiol. **40**: 127-156.
- MARSLAND, D. 1956. Protoplasmic contractility in relation to gel structure: temperature-pressure experiments on cytokinesis and amoeboid movement. Intern. Rev. Cytol. **5**: 199-227.
- MARSLAND, D. A. & D. E. S. BROWN. 1936. Amoeboid movement at high hydrostatic pressure. J. Cellular Comp. Physiol. **8**: 167-178.
- MARSLAND, D. A. & D. E. S. BROWN. 1942. The effects of pressure on sol-gel equilibria, with special reference to myosin and other protoplasmic gels. J. Cellular Comp. Physiol. **20**: 295-305.
- MARSLAND, D. A. & O. JAFFEE. 1949. Effects of pressure on the cleaving eggs of the frog (*Rana pipiens*). J. Cellular Comp. Physiol. **34**: 439-450.
- MARSLAND, D. A. & J. V. LANDAU. 1954. The mechanisms of cytokinesis: temperature-pressure studies in the cortical gel system in various marine eggs. J. Exptl. Zool. **125**: 507-539.
- MAST, S. O. 1926. Structure, movement, locomotion and stimulation in *Amoeba*. J. Morphol. and Physiol. **41**: 347-425.
- MAST, S. O. 1931. Locomotion in *Amoeba proteus* (Leidy). Protoplasma. **14**: 321-330.
- MAZIA, D. & K. DAN. 1952. The isolation and biochemical characterization of the mitotic apparatus of dividing cells. Proc. Natl. Acad. Sci. Wash. **38**: 826-838.
- MAZIA, D. & H. I. HIRSHFIELD. 1950. The nucleus-dependence of P^{32} uptake by the cell. Science. **112**: 297-299.
- MIRSKY, A. E. 1936. Protein coagulation as a result of fertilization. Science. **84**: 333.
- MITCHISON, J. M. 1952. Cell membranes and cell division. Symposium Soc. Exptl. Biol. **6**: 105-127.
- MOMMAERTS, W. F. H. M. 1954. Is adenosine triphosphate broken down during a single muscle twitch? Nature. **174**: 1088-1090.
- MOMMAERTS, W. F. H. M. 1956. The actomyosin system and its participation in organized enzyme reactions. In Enzymes: Units of Biological Structure and Function. : 317-323. O. H. Gaebler, Ed. Academic Press. New York, N. Y.
- MONTGOMERY, E. 1881. Zur Lehre von der Muskelkontraktion. Pflüger's Arch. **25**: 497-536.
- MORALES, M. F., J. BOTTS, J. J. BLUM & T. L. HILL. 1955. Elementary processes in muscle action: an examination of current concepts. Physiol. Revs. **35**: 475-505.
- PARRISH, R. G. & W. F. H. M. MOMMAERTS. 1954. Studies on myosin. II. Some molecular-kinetic data. J. Biol. Chem. **209**: 901-913.
- RHUMBLER, L. 1905. Zur Theorie der Oberflächenspannung der Amöben. Z. Zool. **83**: 1-52.
- SWANN, M. M. 1952. The nucleus in fertilization, mitosis and cell division. Symposium Soc. Exptl. Biol. **6**: 89-104.
- SZENT-GYÖRGYI, A. 1957. Remarks on proteins: summarizing statements. J. Cellular Comp. Physiol. (Suppl.) **49**: 311-316.
- TS'o, P. O. P., L. EGGMAN & J. VINOGRAD. 1956. The isolation of myxomyosin, and ATP-sensitive protein from the plasmodium of a *Myxomycete*. J. Gen. Physiol. **39**: 801-812.
- WAUGH, D. F. 1957. A mechanism for the formation of fibrils from protein molecules. J. Cellular Comp. Physiol. (Suppl.) **49**: 145-164.
- WEBER, H. H. 1955. The link between metabolism and motility of cells and muscles. Symposium Soc. Exptl. Biol. **9**: 271-281.
- WEBER, H. H. 1957. The biochemistry of muscle. Ann. Rev. Biochem. **26**: 667-698.
- ZIMMERMAN, A. M., J. V. LANDAU & D. A. MARSLAND. 1957. Cell division: a pressure-temperature analysis of the effects of sulfhydryl reagents on the cortical plasmagel structure and furrowing strength of dividing eggs (*Arbacia* and *Chaetopterus*). J. Cellular Comp. Physiol. **49**: 395-435.
- ZIMMERMAN, A. M., J. V. LANDAU & D. A. MARSLAND. 1958. The effects of adenosine triphosphate and dinitro-o-cresol upon the form and movement of *Amoeba proteus*; a pressure-temperature study. Exptl. Cell Research. **15**: 484-495.

SYNCHRONIZATION OF CELL DIVISION IN AMOEBAE*

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Synchronization of cell division has recently been produced or noted in a variety of cell types.¹⁻⁸ This report on *Amoeba proteus* includes some of the earlier work⁹ mentioned in the publications of other investigators^{10, 11} and in our current work. With due apologies to *A. proteus*, most of our present work is done on other cell types; however, the enchanting behavior of this species would never permit us to forsake it.

General Considerations and Early Work

Before beginning a discussion of the synchrony of cell division it would be profitable to define terms. Almost everyone would accept the term synchronous cell division to mean the simultaneous division of cells. To synchronize cell division would mean that this simultaneous division process is controlled by some type of active interference with the normal random behavior of this process. Granted that this is possible, it then becomes important to specify how well the process has been controlled. It is obvious that cells that have a short generation time relative to the time that division figures can be identified will have a high mitotic index. For example, if the mitotic time is equal to half the generation time, one should see approximately 44 per cent of the cells in division at any given moment. In the case of *A. proteus*, which has a generation time of 24 hours and a mitotic time of one-half hour at 23° C., about one forty-eighth of the cells should be in division at any one time, giving a mitotic index of about 2 per cent. The normal mitotic index is a statistic that cannot be ignored when evaluating the effectiveness of any synchronizing procedure. Consequently, it would seem reasonable to specify how well division is controlled by a plot of division frequency per unit of time as a function of time. From the distribution of areas one can then recognize the departure from the rectangular distribution seen with a constant mitotic index.

The acceptability of this criterion may well hinge on the use to which the synchronized cells are to be put. The uses of synchronized cells fall into two general categories: first, the study of cell division or, specifically, the mitotic processes; and second, the over-all process of growth and division with emphasis on the processes that accompany individual cell growth. Synchronized cells with a high division index would thus provide a visual source of confidence for an investigator interested in the mitotic processes, whereas an extended interphase might be desirable for studies on the variety of syntheses that accompany growth. These considerations cannot be too rigorously held, since there is little certainty that in synchronized cells the syntheses that accompany cell growth will remain, of necessity, in phase with the mitotic process. The foregoing remarks are made more in defense of the rather poor synchrony that

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has been found in *A. proteus* than as a criticism of the excellent methods that produce cells in which 90 per cent of the divisions are visible at one time.

In the initial experiments on the synchronization of division in *A. proteus* the purpose was to define a standard cell. It was obvious that, in observing any particular amount of a constituent in, or activity of, a single cell, it would be seen to vary by a factor of two, depending on whether a daughter or a preprophase cell was analyzed. Since the use of single cells for certain analyses is limited, a means was sought that would provide an appreciable number of amoebae in the same stage of growth. There are unsupported rumors that cells in the onion root tip and the *Opalina* have a higher mitotic index at night than during the day. In the latter case the organism is an intestinal parasite to cold bloods; consequently, the only direct external cue upon which this organism could phase its activity is the diurnal change in temperature.

The work of Ephrussi¹² has clearly shown that the temperature coefficients of each stage in the mitotic process are different, and Daniel and Chalkley¹³ have shown that the stages of division in *A. proteus* have distinctly separable activation energies. A temperature cycle was designed that would in effect imitate, in a conservative way, the diurnal fluctuations of temperatures normally found in nature and so that the generation time of the cells on the sequence of temperatures would be equal to the phase of the repeating cycle, namely 24 hours. The range of temperature change was set to pass through one of the breaks in the activation energy curves found by Daniel and Chalkley.¹³ The generation times of *A. proteus* grown on *Tetrahymena pyriformis* at 18° and at 25° C. were determined, and these data indicated that approximately 1 division every 24 hours could be expected on a cycle of 12 hours at 18° and 12 hours at 26° C. The first experiments on this cycle were good. Clones remained in synchronous division for seven generations, the divisions occurring after the transition from 18° to 26° C. Unfortunately, results of this kind on mass cultures were not consistently obtained and, despite the fact that on a number of occasions the precision of synchrony was high, on other occasions complete dissynchrony of the cultures was manifest. These findings gave cause to refrain from publishing.

Conditions and Mechanisms

The task of understanding and improving this system was undertaken rather than that of producing cells for analysis. It was recognized that, if a temperature change of any kind was used, the treatment of necessity would perhaps alter the cell's synthetic rate in a way that might destroy the patterns that were being sought. This procedure would be comparable to changing more than one variable at a time in an experiment. Three general areas of concern were considered to be important to this undertaking: nutritional, ecologic, and cytological.

To date there has been little recorded success on culturing *A. proteus* in an axenic medium. Washed food organisms, however, have been used by some workers^{14, 15} and, at the suggestion of C. B. Van Neal, we instituted the use of washed *Tetrahymena*¹⁶ under nonsterile conditions. It is felt that a well-defined

sterile medium would go far toward improving the synchrony of division in this organism.

To define better the conditions under which the synchronization could be carried out, experiments on the variation of the generation time as a function of temperature were performed. The amoebae were obtained originally from H. Hirshfield and clone cultures were used routinely. The cultures were grown on an agar base in 50-mm. Petri dishes with ample supplies of washed *Tetrahymena* in a modified Hahnert's medium.¹⁷ The incubation was carried out in a 4° C. cold room with a series of incubation cabinets that were set to work against the background temperature. Temperatures from 10° to 30° C. \pm 1° C. were used, and counts were made in the vicinity of the incubator to avoid disturbing the cultures any more than was necessary to make the observation. Continuous attachment to the agar surface was a prerequisite to a consistent growth picture. Counts were made at intervals that were less than the estimated generation time at the particular temperature. They were carried out in subdued light. FIGURE 1 shows the plot of generation time against temperature obtained. The divisions were very consistent and growth rates were constant over the initial period of growth. In the 10° C. cultures, where growth was expected to be abnormal, divisions occurred every $4\frac{3}{4}$ days in a completely normal fashion. At 30° C. growth was normal, but detachment from the agar surface was more frequent. Higher temperatures gave rates that were completely inconsistent.

FIGURE 2 is the Arrhenius plot of these data. It can be seen that a break occurs in the vicinity of 20° C. The activation energy between 20° and 30° C. is 11,500 calories, and 21,000 calories from 20° to 10° C. It is interesting to compare these values with those found by Daniel and Chalkley¹³ for various stages of the division process. TABLE 1 lists some of these values, the values calculated from the feeding data on *A. proteus* from Mast and Fennell,¹⁴ and movement data of Mast and Prosser.¹⁸

The use of different activation energies of various cellular processes as a foundation for studying the synchronization behavior is very attractive. However, it should be recognized that the meaning of this function is obscured when applied to complicated systems that, like the processes of growth and division, consist of both simultaneous and sequential reactions that may be coupled in a variety of ways. The validity of this fact can be appraised by referring to the values of activation energies for some of the cellular processes in *A. proteus*. It can be seen in TABLE 1 that the value found for the over-all mitotic process is intermediate with respect to the subprocesses of mitosis. This might be expected for truly sequential systems, but formal proof of this fact is a formidable task. Food capture and amoeboid movement give values that vary gradually with increasing temperature, going negative in the region of 26° C. The over-all growth and division shown in FIGURE 2 could also be interpreted in this way except for the value in the region of 20° C. which might be used, as we have done, as a breaking point, thus giving two different activation energies. Its departure could be assumed to be an error, and a smoothed curve would result. If the relationship between the activation energies of over-all processes and

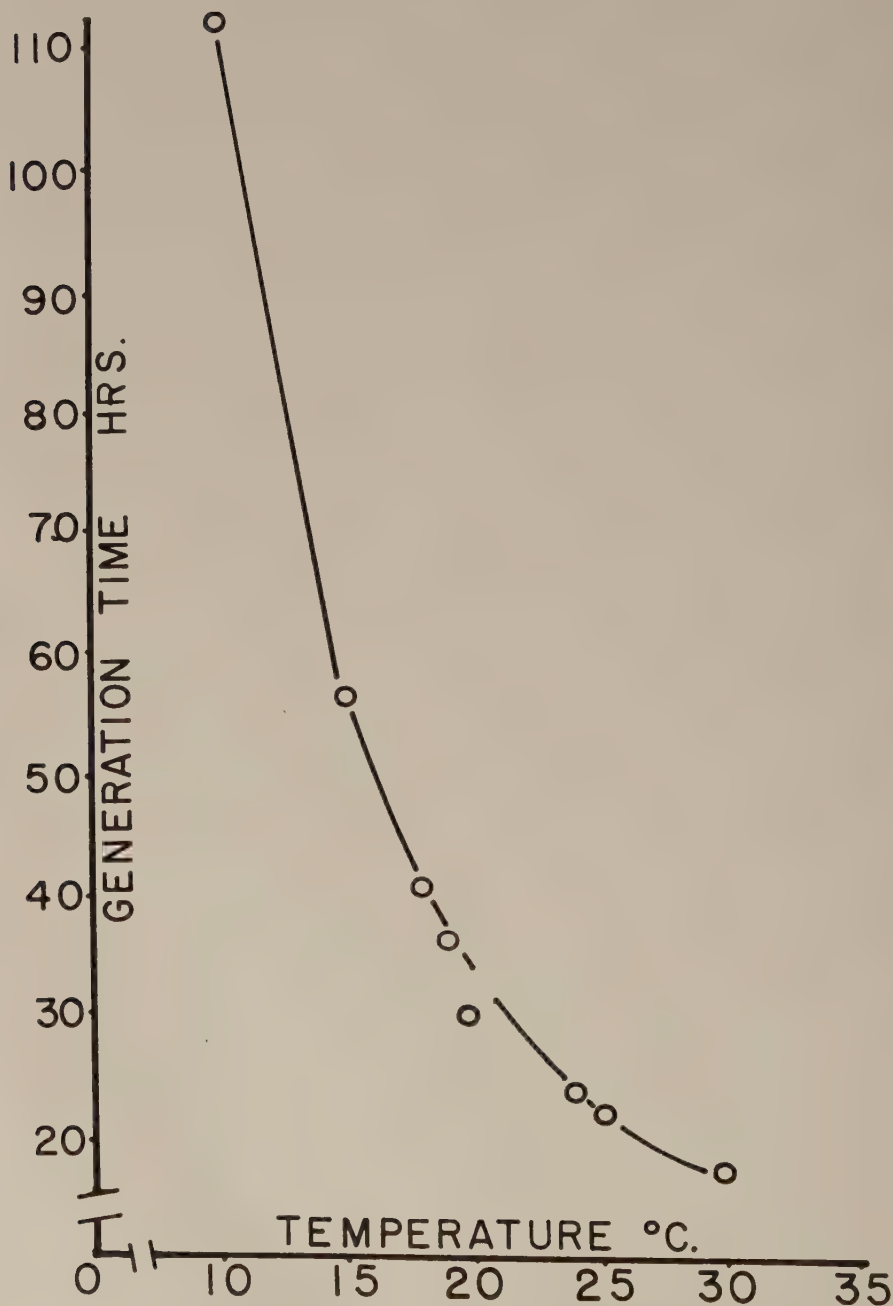


FIGURE 1. Generation time of *A. proteus* at different incubation temperatures.

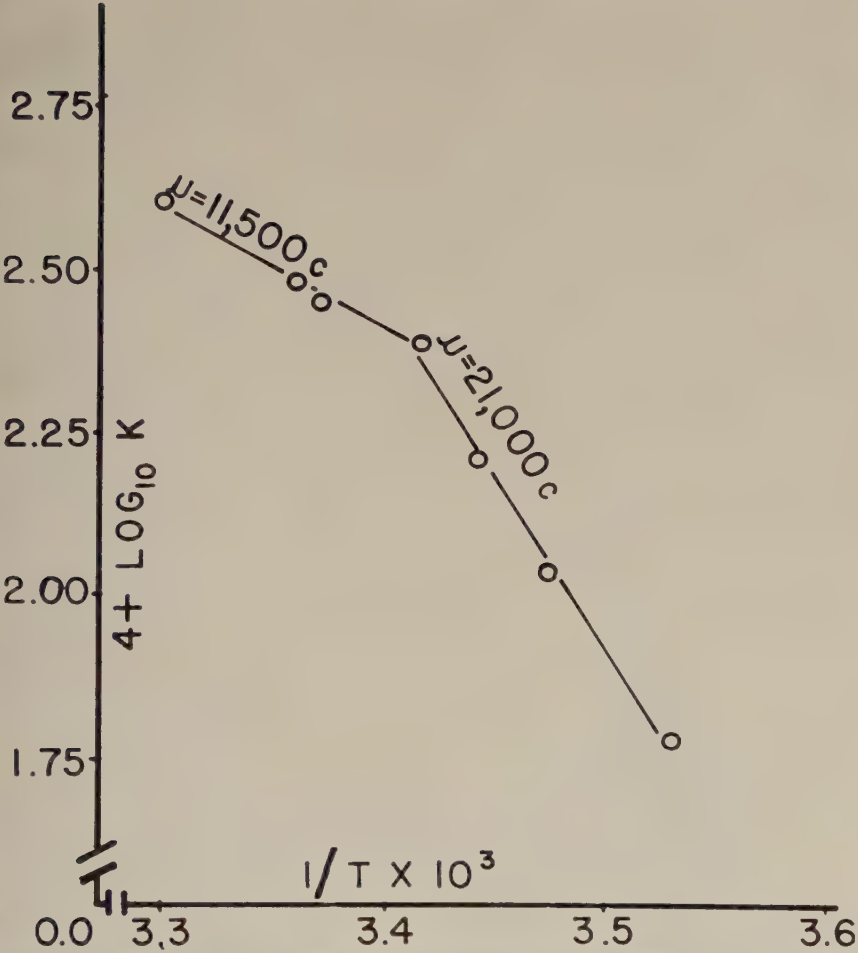


FIGURE 2. Arrhenius plot of over-all growth and division (generation time) of *A. proteus*

TABLE 1
ACTIVATION ENERGIES OF VARIOUS PROCESSES IN *Amoeba proteus*
RANGE OF TEMPERATURE: CENTIGRADE

	30°	25°	20°	15°	10°	5°	
Mitosis (over-all)		26°		16,500 calories		6°	Workers
Prophase		26°		11,700	13°		Daniel and Chalkley ¹³
Anaphase		26°		20,200	13°		Chalkley ²⁶
Nuclear division		26°		16,600		6°	Chalkley ²⁶
Cytoplasmic division	30°	7,300	21°	20,500	11°		Chalkley ²⁶
Food capture	30°	0	25°	18,000	15°		Estimated from Mast and Fennell ¹⁴
Amoeboid movement	30°	0	25°	0 to 18,000	15°		Estimated from Mast and Prosser ¹⁸
Growth and division	30°	11,500	20°	20,900	10°		James

subprocesses is always intermediate, in this instance we should expect that the activation energy for the over-all growth and division process would result from an average of high values for mitosis and low values for feeding in the region of 26° C.

One argument on the mode of action of a temperature cycle in aligning the cellular processes might be as follows. At 18° C. food capture and amoeboid movement both have activation energies of approximately 18,000 calories; over-all mitosis has a value of 16,500 calories. Thus, 18° C. would favor mitosis only slightly, if at all. At 26° C., feeding and movement are maximal with activation energies approaching zero, while over-all mitosis still requires 16,500 calories. This range would favor feeding over mitosis. Thus, it might be expected that divisions would occur during the latter part of the cold period, except for the fact that anaphase and cytoplasmic division appear to have higher requirements. This scheme can be seriously questioned because of the neglected time factor, namely, the fact that the rates and duration of each of the sequential processes are not open to direct evaluation. Since the duration of each process is as important as the energy barriers that presumably must be surmounted, consideration of activation energies gives us little more than a vague suggestion of how a pattern of reactions might be ordered by a temperature cycle. A strong argument against the role of different activation energies in synchronization of cell division by temperature is equally difficult to make, for, even if one assumes that a master reaction controls the over-all processes, its inhibition or the inactivation of its enzyme by temperature still follows the same general concept that temperature is acting as a valve that permits certain reactions to continue while inhibiting others. The early experiments with clone cultures on a temperature cycle of 12 hours at 18° and 12 hours at 26° C. showed a tendency to divide at the beginning of the warm period. It is interesting to note that, if one assumes that the generation time can be fractionated and that any fraction of it at a given temperature is equal to any other fraction, a program of temperatures and times using the generation times of FIGURE 1 would require 12 hours at 18° C. and 12 hours at 30° C. to give a division every 24 hours. Our cycle maintains one division every 24 hours at a lower temperature range, namely, 18° and 26° C., implying that the generation time cannot be fractionated and that the system is more effective on a cycle than would be predicted from the generation time data.

FIGURE 3 is an observation made on 50 cells taken from a mass culture maintained on the 18° and 26° C. cycle for 5 days. The cycle is shown at the top, while a histogram of the number of divisions on a per-hour basis is presented below. As can be seen, divisions are maximal about 3 hours after the end of the cold period. The maximum mitotic index is low, not exceeding 20 per cent. Recent use of a 16-mm. time-lapse movie camera gives very similar results. This time-lapse technique suffers from one shortcoming, namely, the difficulty of maintaining high resolution together with sufficient field size. Its use has shown that division figures are most numerous during the early warm period and that other temperatures and periods can be used if both the periods and the temperatures are selected so that a generation is completed in phase with

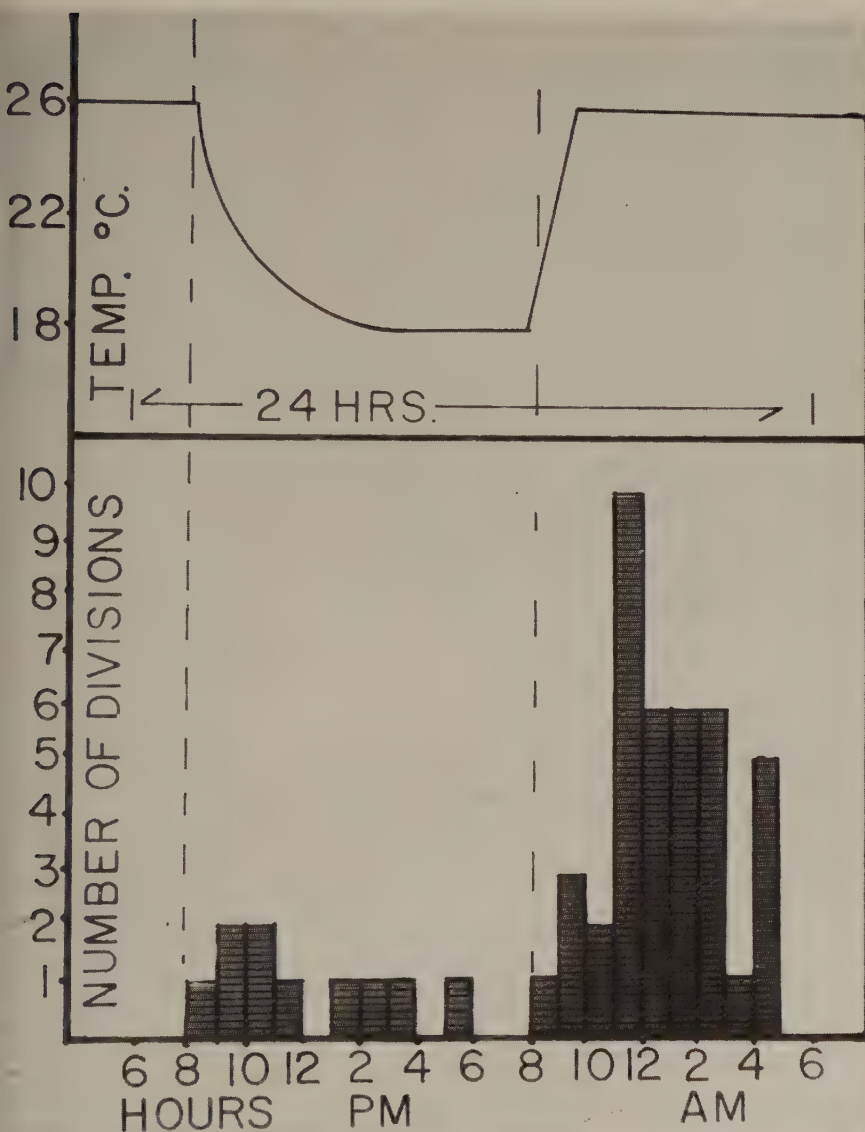


FIGURE 3. Cell divisions per hour in a random sample of 50 *A. proteus* from a mass culture maintained on a temperature cycle (above).

the cycle. Temperatures that are too low cause a progressive lag in the period during which divisions occur, while the opposite happens with temperatures that are too high. The extent of this effect is worthy of further examination. Time-lapse methods have also shown that the pairing of *A. proteus* occurs in a

characteristic manner. Two cells often come into contact and remain in close proximity for a short period of time. This may be accidental or have other significance. This observation is proof that characteristic paired cells do not of necessity mean, as might be assumed, that cell division has recently taken place.

Studies with the Interference Microscope

This cyclical response of the growth and division of the cell to temperature can be approached also as a phenomenon that should be observable at the cytological level. To test this possibility, studies of the effects of temperature changes on the cytoplasm and nucleus by use of an interference microscope have been initiated. The following findings are preliminary and, since single cells are used, variations from one cell to another are the rule rather than the exception. One of eight sets of observations is used to illustrate our findings. It was recognized that to make such studies reproducible the amoeba must be compressed and maintained at a fixed thickness for an extended period. Lack of reproducibility might result from a number of factors. The most obvious is the injury that might be sustained by the cell due to excessive compression. Less apparent is the possibility that the cell might show heterogeneity with respect to its refractive index along the axis of compression. The latter possibility would lead to measurements that would not remain consistent as the degree of compression changed. Compression of *A. proteus* to less than $15\ \mu$ in thickness often leads to cytolysis. Compression to around $25\ \mu$ exposes the nucleus as a disk with a small layer of cytoplasm surrounding it. Petroff-Hauser bacterial counting chambers are made with a depth of $20\ \mu$. However, accurate measurements of the chamber depth by a spherometer (reading to $2\ \mu$) for rough estimation and by the interference microscope for finer measurements were necessary. The latter method consisted of counting the fringes at a glycerine-water boundary and measurement of the fractional difference between the last fringe and the second-to-last fringe. These measurements can be made to an accuracy of at least one tenth of a wave length, but rapid photographic methods must be used because mixing of the two phases occurs. However, since we have found that replacement of the cover glass is reproducible only to within $0.5\ \mu$, our chamber depth of $23.5\ \mu$ is considered good to within those limits using an optical-quality thick cover glass. The chamber was provided with a transparent temperature jacket of such nature that the cell could be cooled or warmed by circulating water beneath the area under observation. Water of the desired temperature was circulated in this apparatus from a water bath. The thickness of the entire apparatus was such that $100\times$ magnification could be used with only a slight loss in image quality. Pictures of cells in this chamber were taken with an Ibsco microcamera* in conjunction with the Baker-AO interference microscope. Calibrations of the field size were made directly from the field square on the Petroff-Hauser chamber. One square is $50\ \mu$ on an edge. Temperatures below 10°C . were not used and the upper range was limited to temperatures of not more than 37°C .

* Product of E. Leitz Co., Inc., Wetzlar, Germany.

As a means of checking the validity of the method, cells were placed in the chamber and the various interferometric determinations made, namely, determining the retardation of various parts with respect to water. Photographs were taken at the same time with the analyzer set to give the desired contrast of the part under consideration. Since the thickness of the cytoplasm is set by the depth of the compression chamber and since the area is available from the photographs, the cell volume independent of the interferometric constants was obtained. The areas were determined by planimetry. The restrictions that are placed on the thickness of the amoeba due to considerations previously mentioned require that our chamber have a depth of not less than $20\ \mu$. This thickness of amoeba cytoplasm will give a retardation of one full wave length if its refractive index is 1.350 or greater. Since the cytoplasm of most cell types has a refractive index of this magnitude,¹⁹ it was recognized that our angular measurements would be the result of second-order retardation, meaning that our retardations would fall somewhere between 360° and 720° of arc. A careful check for a boundary fringe reversal at the edge of the amoeba does not readily reveal this to be the case. The edges of contractile vacuoles, however, do show this reversal. See FIGURE 4. However, evaluation of the dry mass, assuming a first-, second-, and third-order retardation, discloses values of 0.05, 0.24, and $0.43\ \mu\text{g.}$ per cell, respectively. The second-order value agrees closely with the actual dry mass found for *A. proteus* by Cohen²⁰ and the value published by Mellors²¹ for the weight per unit volume of tissue culture cells. To our knowledge this is the first time that interferometric anhydrous mass has been directly compared with dry weights obtained by conventional means. Determinations of anhydrous mass and refractive index of the cytoplasm and nucleus were carried out by methods similar to those introduced by Mellors²¹ and Faust.²² These values are given in TABLE 2. The results of a typical experiment on the effect of temperature on the anhydrous mass of the nucleus are also given in this table. The procedure was as follows. The cell was placed in the chamber and allowed to come to equilibrium with the temperature of the jacket, usually 22°C. At zero time the analyzer was set to give maximum darkness for, first, the background, then the cytoplasm and, finally, the nucleus, and the angles were recorded. A photograph was then taken. This was done at several intervals over the first 20 min. The temperature was then lowered to 10°C. , where it was allowed to remain for about 15 min. Measurements at the low temperature were made, but are not reported. Then it was returned to 22°C. and new measurements were made. It can be seen that the cytoplasmic volume and apparent mass tend to increase. This is most probably due to the water exchange, since increase in vacuolar activity is noted. Of course, there is no sound reason for a net increase in mass, although a molecular optical phenomenon cannot be ruled out. Sol-gel considerations may also be important in this effect. If the vacuolar volumes were subtracted from the total volume, closer agreement would be seen between the anhydrous mass of the cytoplasm before and after treatment. Accompanying this effect is a definite decrease in the anhydrous mass of the nucleus which, in this case, shows a loss of about $1000\ \mu\text{g.}$ as shown in TABLE 2. This appears to be a homogeneous loss, and

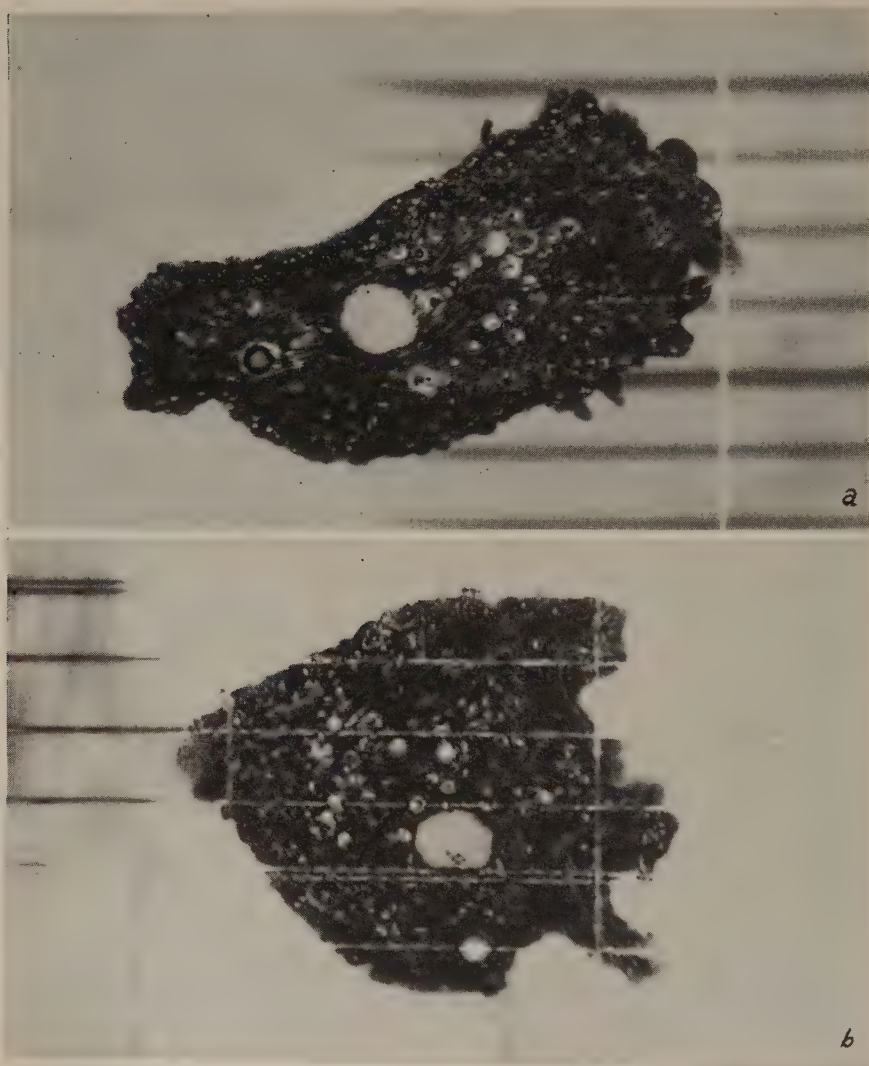


FIGURE 4. Photographs of *A. proteus* using an interference microscope. (a) Amoeba in the compression chamber before cold treatment. (b) The same amoeba after cold treatment.

may be followed by a slight volume decrease. It is found also when the cells are heat-treated to 35°C . or when cytoplasm is amputated. This is perhaps related to the volume reduction of the nucleus following cytoplasmic amputation found by Gruber²³ and more recently studied by Prescott.²⁴ The general appearance of the cells before and after treatment (FIGURE 4) is not significantly different. The slight change in nuclear area may readily be seen. The texture of the cytoplasm is similar in both pictures, but they show that maximum

TABLE 2
EFFECT OF TEMPERATURE CHANGE ON CELL PROPERTIES

	Time (min.)	Temp.	Area μ^2	Vol. μ^3	Anhydrous mass
Cytoplasm	0	22° C.	6.11×10^4	14.4×10^5	0.240 $\mu\text{g.}$
Nucleus	0	22° C.	0.21×10^4		$9.90 \times 10^3 \mu\text{g.}$
Cytoplasm	8	22° C.	6.07×10^4	14.3×10^5	0.240 $\mu\text{g.}$
Nucleus	8	22° C.	0.21×10^4		$9.90 \times 10^3 \mu\text{g.}$
Cytoplasm	15	22° C.	6.20×10^4	14.6×10^5	0.240 $\mu\text{g.}$
Nucleus	15	22° C.	0.22×10^4		$10.00 \times 10^3 \mu\text{g.}$
Cytoplasm	20	22° C.	6.09×10^4	14.3×10^5	0.242 $\mu\text{g.}$
Nucleus	20	22° C.	0.22×10^4		$10.00 \times 10^3 \mu\text{g.}$
Cytoplasm	25	10° C.	No measurements—cold shock		
Nucleus	40	10° C.	No measurements—cold shock		
Cytoplasm	45	22° C.	6.79×10^4	15.9×10^5	0.260 $\mu\text{g.}^*$
Nucleus	45	22° C.	0.19×10^4		$8.70 \times 10^3 \mu\text{g.}$
Cytoplasm	80	22° C.	7.00×10^4	16.4×10^5	0.270 $\mu\text{g.}^*$
Nucleus	80	22° C.	0.20×10^4		$9.00 \times 10^3 \mu\text{g.}$

Refractive index for cytoplasm, 1.363; for nucleus, 1.369.

* Not corrected for vacuoles.

retardation of the cytoplasm does not include all of the granules and, consequently, gives an average mass for the ground substance only. The possibility that a nuclear component may leave the nucleus with temperature treatment may provide a mechanism by which synchrony is initiated. Loss of a component may set nuclear synthesis of all cells back to a common starting point. This is a condition resembling behavior seen in other cells.²⁵

Diurnal Effects Accompanying Daylight and Darkness

Another aspect of these investigations was revealed in part by some preliminary pictures taken with the time-lapse arrangement. Stock amoeba cultures maintained in a north window were known to show a marked periodic difference in activity. On a number of occasions it was noted that after dark the entire culture showed cells in a rounded configuration that were not predivision forms as noted by Chalkley.²⁶ These cells appear attached, but without major pseudopodial processes. At dawn they immediately assumed a streaming form, but without the directional effects normally seen with strong light. Since we were interested in the diurnal aspects of cellular activity, a recording of the north window daylight was made and time-lapse pictures were taken over the period of light and dark. It was noted that the maximum daylight at the level of the culture shelf on a bright sparsely cloudy day was very near to 300 foot-candles. A recording of light intensity coming from the north sky over a period bracketing the daylight hours is shown in FIGURE 5. The waviness is due to passing cumulus clouds. Photographic recordings were made of the culture over the period of light and dark, and the general state of the cells was noted. The lower portion of FIGURE 5 shows three photographic frames taken of the same field of the culture during early morning darkness, daylight, and evening darkness. This was done at room temperature which, at this time of the year, was fluctuating around 24° C. The same configurations are seen for

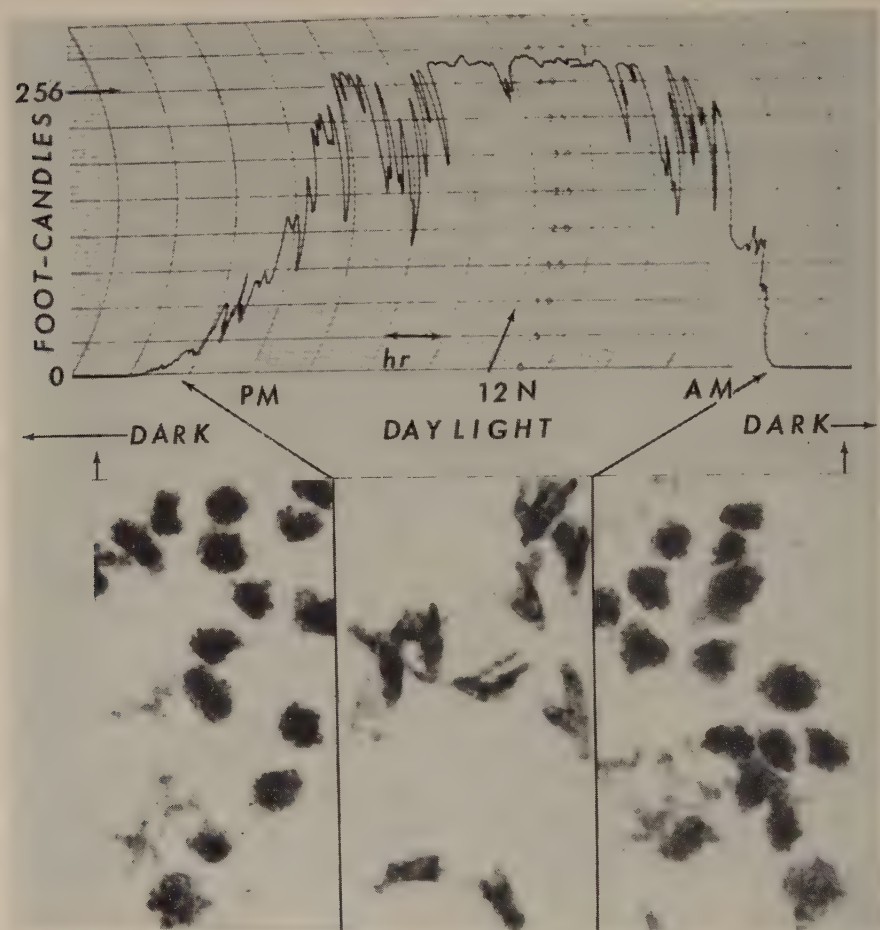


FIGURE 5. Three representative frames of time-lapse photographs of *A. proteus* in a culture exposed to the natural light cycle shown above.

all regions of the culture within the time limits indicated by the lines. The transition from quiescent to active phase appears to have a threshold of about 200 foot-candles and takes about 10 min. The reverse transition takes slightly longer, from 20 to 30 min. This has not been examined well enough to be certain of the relationship between threshold, temperature, and the state of the culture. In the present observations only young cultures were used. In old cultures the organisms do not enter the quiescent state. Feeding appears to be essential to this phenomenon. The light used to take the photographs was of far too short a duration to have any effect on the configuration. We have also produced this effect at will by the use of darkness and artificial light, namely, a tungsten light of an intensity from 200 to 250 foot-candles. The temperature

was held constant at 22° C. It is interesting to note that this range of intensity is that commonly found in open shade and is slightly higher than that necessary to view cultures with a dissecting microscope. This may explain why such an obvious phenomenon, to the best of our knowledge, has not been previously mentioned in the literature. The fact that intense light will inhibit division is well known yet, at this intensity, the only cells that do not go into the streaming phase are those that are well-formed division spheres.²⁶

From the rather diverse data presented, it appears that what success we have had in the synchronization of cell division in *A. proteus* can be attributed in part to a somewhat obscure relationship between cell division and diurnal factors. We are tempted to emphasize these relationships by suggesting that they belong to a realm that could be called "the ecology of cell division."

The synchronization of division in *Tetrahymena* by Scherbaum and Zeuthen¹ is probably one of the most dramatic proofs that temperature procedures can be successful, but the method can hardly be construed as having an ecologic basis. This use of a series of short supraoptimal temperature shocks, followed by a period at the lower temperature that ends with division, can be interpreted as illustrating two facts: first, the shock is operating on a portion of the cell cycle that is short relative to the generation time; and, second, repetitions of the shocks tend to collect these sensitive periods, which occur at random in time, into one period. This approach may work on *A. proteus*, but would be difficult to test until its nutrition has been better defined. The procedure we have used on *A. proteus* from the beginning is the use of a repeating daily cycle in phase with the generation time that we believe, by reason of its repetition, also tends to localize the division in time. Parenthetically, we have obtained better results applying this method to culturing the flagellate *Astasia longa*. The main difficulty in using this method results from our inability to maintain a culture under constant conditions over many generations in a manner comparable to that obtained by chemostatic culture methods.

Our attention is drawn further to the diurnal aspects of cell division by the recognition that there is a response of the cell to light at normal ecologic levels of daylight. The exact interaction of light and temperature is not known. One would guess that feeding may be increased in light, although feeding in light and dark has not been measured. Since light in nature generally will be associated with the higher temperature, one would expect that division would occur at the end of the dark cool period. However, we have seen that, in our study with temperature alone, divisions occur in the early to middle-warm period. We are now experimenting with light cycles at constant temperature, and we also hope to combine temperature and light cycles. One of the most striking examples of light effects found in nature is that examined by Pirson,²⁷ who has placed the coenocytic algae *Hydrodictyon* on light cycles and observed that both photosynthetic rate and respiration not only undergo smooth cyclical changes, which show maximum at specific times independent of the constant conditions over each portion of the cycle, but also persist even after removal from the cycle. This organism is not a true cell, but undergoes a doubling of constituents in phase with the light cycle. This system is relatively tempera-

ture-independent, as is the behavior of *Gonyaulax* which, as found by Sweeney and Hastings,⁸ shows a correlation between growth and division and the normal diurnal light cycle. The temperature independence of the generation time is less marked in *Chlorella* cells, which have been synchronized and analyzed by Iwamura.⁶

Cells in their variety of types and activities have appeared to explore, in the evolutionary sense, every device that might contribute to steady-state behavior. Steady-state behavior is at once the state of minimum entropy production and an evolutionary direction. Amoebae, by the criterion of Prigogine and Wiame,²⁸ would be more primitive in the thermodynamic sense than cells that show a marked temperature independence of their generation time. The means by which light and temperature changes could come to be reflected as patterns of cellular activity, growth, and division depend in part on how primitive this diurnal phenomenon may be. Certainly one can look upon it as a response of the cell, as a complex organic unit with a variety of internal feedback controls, to the persistent and repetitive action of all the parameters that change with day and night; as a more fanciful alternative, one may look upon it not as a response but as the remnant of a primitive system, a system that in some cells has insufficient complexity for control. Thus, these cells have no means of freeing themselves from the thermodynamic constraints imposed by nature.

References

1. SCHERBAUM, O. & E. ZEUTHEN. 1954. Exptl. Cell Research. **6**: 221.
2. HOTCHKISS, R. D. 1954. Proc. Natl. Acad. Sci. U. S. **40**: 49.
3. NEFF, R. J. 1955. Terminal Rept. Public Health Research Grant E-623 (M and G).
4. BRUCE, V. G., K. G. LARK & O. MAALOE. 1955. Nature. **176**: 563.
5. BARNER, H. D. & S. S. COHEN. 1955. Federation Am. Soc. Exptl. Biol. Proc. **14**. (Abstr.)
6. IWAMURA, T. 1955. J. Biochem. **42**: 575.
7. HUNTER-SZYBALSKA, M. E., W. SZYBALSKI & E. D. DELAMATER. 1956. J. Bacteriol. **71**: 17.
8. SWEENEY, B. M. & J. W. HASTINGS. 1958. Plant Physiol. **32**: 25.
9. JAMES, T. W. 1954. Ph.D. Thesis. Univ. Calif. Berkeley, Calif.
10. MAZIA, D. 1956. Am. Scientist. **44**: 1.
11. MAZIA, D. & D. M. PRESCOTT. 1954. Science. **120**: 120.
12. EPHRUSSI, B. 1926. Protoplasma. **1**: 105.
13. DANIEL, G. E. & H. W. CHALKLEY. 1933. J. Cellular Comp. Physiol. **2**: 311.
14. MAST, S. O. & R. H. FENNELL. 1938. Physiol. Zool. **11**: 1.
15. ANDRESEN, N. 1956. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **29**: 435.
16. PRESCOTT, D. M. & T. W. JAMES. 1955. Exptl. Cell Research. **8**: 255.
17. HAHNERT, W. F. 1932. Physiol. Zool. **5**: 491.
18. MAST, S. O. & R. H. PROSSER. 1932. J. Cellular Comp. Physiol. **1**: 333.
19. ROSS, K. F. A. 1954. Nature. **174**: 836.
20. COHEN, A. I. 1957. J. Biophys. Biochem. Cytol. **3**: 923.
21. MEHLORS, R. C., A. KUPFER & A. HOLLENDER. 1953. Cancer. **6**: 372.
22. FAUST, R. C. 1956. Quart. J. Microbiol. Sci. **97**: 569.
23. GRUBER, K. 1912. Arch. Protistenk. **25**: 316.
24. PRESCOTT, D. M. 1956. Exptl. Cell Research. **11**: 94.
25. SUMMERS, L. G., T. W. JAMES & B. H. LEVEDAHL. 1956. 26th Ann. Meeting W. Soc. Nat. :8 (Abstr.).
26. CHALKLEY, H. W. & G. E. DANIEL. 1933. Physiol. Zool. **6**: 592.
27. PIRSON, A. 1952. Ber. deut. botan. Ges. 65, Schlussheft, **7**: 8 (Abstr.).
28. PRIGOGINE, I. & J. M. WIAME. 1946. Experientia. **2**: 451.

PINOCYTOSIS IN AMOEBAE*

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Warren Lewis first described the uptake of fluid droplets by cells in 1931.¹ He coined the word "pinocytosis" and, with remarkable insight, sketched out many of the implications that concern us today. Mast and Doyle described similar activity in amoebae in 1934,² but interest in this curious process has become widespread only in the last few years.

Most of the experimental studies on pinocytosis have come from the laboratory of Heinz Holter, who reviews the field elsewhere in these pages. My own remarks will be limited to a brief summary of two recent studies on the mechanisms of pinocytosis in amoebae and to an account of some work we are doing now along the same line, using mass cultures of the large amoeba, *Chaos chaos*. Before going further I must express my appreciation to Holter for introducing me to the amoebae and for suggesting, some years ago, that their drinking habits should be investigated.

One of the remarkable things about pinocytosis in amoebae is the fact that a variety of salts, proteins, and proteinaceous materials are capable of inducing the drinking response.^{2, 3, 4} Serum gamma-globulin is one such protein. Holter and I found that rabbit gamma-globulin labeled with fluorescein is taken up in sizable amounts by *C. chaos*.³ We found that the amount taken up is uniform among cells of similar size under controlled conditions, and we studied some of the changes that occur in the pinocytosis droplets after they are engulfed. However, we obtained no direct evidence showing how the process begins or what mechanisms are responsible for the formation of pinocytosis channels and droplets.

Two studies have been completed recently that deal with these problems of mechanism. One was done by Verne Schumaker⁵ in Brachet's laboratory in Brussels, Belgium, and the other by Philip Brandt⁶ in my laboratory at the University of Pennsylvania. Schumaker followed the uptake of two proteins by *Amoeba proteus*. He labeled ribonuclease and cytochrome *c* with radioactive iodine and measured protein uptake in a series of experiments as a function of time, concentration of protein, temperature, pH, and metabolic activity.

Schumaker was able to distinguish three stages in the uptake of protein. In the first, protein is bound to the membrane or cell surface. This binding is reversible, is a function of the concentration of protein in the medium, is largely independent of pH or temperature, and is not greatly affected by meta-

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bolic inhibitors. A second stage begins when protein binding exceeds a certain level. This is marked by a sudden increase in binding, as though new binding sites become available. This stage, like the first, seems insensitive to metabolic inhibitors, to pH , and to cooling. By contrast, the third stage of uptake is sensitive to inhibitors and to cooling. Bound protein is no longer in equilibrium with protein in the medium. This stage Schumaker interpreted as the ingestion of membrane, bearing bound protein.

Brandt approached the same problem cytologically, first using fluorescent gamma-globulin to follow pinocytosis in *C. chaos*. He began by examining amoebae very shortly after they were taken out of the labeled protein and washed. FIGURE 1 is a fluorescence photomicrograph of such a washed amoeba, flattened beneath a cover slip. Little detail can be seen in the thicker parts, but near one edge it can be seen that the plasmalemma is as brightly fluorescent as the droplets already taken up. More detail could be made out in sections cut after freeze-drying the amoebae. FIGURE 2 illustrates the fluorescence pat-



FIGURE 1. Fluorescence photomicrograph of an intact amoeba after treatment with fluorescent globulin and brief washing.



FIGURE 2. Fluorescence photomicrograph of an amoeba after treatment with fluorescent globulin and brief washing. Fixation by freeze-drying, section thickness $1\ \mu$.

tern in a section of an amoeba that had been left for one half hour in labeled globulin.

Since there are possibilities for artifacts in the direct use of fluorescent proteins, Brandt repeated and extended his studies by using unlabeled gamma-globulin from chicken serum. This protein could be detected in sections of freeze-dried amoebae by applying a fluorescent rabbit antibody specific for the chicken globulin. As controls, other sections were treated with a fluorescent globulin solution prepared from normal rabbit serum.

Using these indirect techniques, Brandt studied the effects of variations in protein concentration, time, ionic strength, pH , and temperature, on the uptake of chicken gamma-globulin. For details of these experiments, as for details of the work of Schumaker, the reader is referred to their publications. In the main, the fluorescence methods gave results entirely consistent with Schumaker's interpretation, and focussed our attention on the plasmalemma.

Bairati and Lehmann⁷ had reported that the slime coat of *A. proteus* reacts positively to the periodic acid-Schiff (PAS) stain. Brandt found this to be true for *C. chaos*, as well, and discovered that pinocytosis droplets could be differentiated from the many other types of vacuoles in the cytoplasm by the positive PAS reaction of their limiting membranes. Amoebae were treated with chicken globulin, washed, fixed, and sectioned. Adjacent sections were stained by the PAS technique and by the fluorescent antibody technique. The result showed a good correspondence between the distribution of bound protein and that of the mucoid material.

From other observations that Brandt made on living amoebae, by phase-contrast microscopy, it appears that the drawing in of channels and the "pinching off" of channels or droplets from the plasmalemma depends upon the attachment of the plasmalemma at many points to the underlying gel. Engulfment seems to follow directly from the normal process of gel contraction and sol extrusion. If this interpretation is correct, the crucial point in the pinocytosis mechanism occurs in the first stage when the binding of proteins or other ions alters the physical state of the plasmalemma.

Both Schumaker and Brandt discuss some aspects of this matter in their papers, but I think it can hardly be settled until we know much more about the nature of the binding sites. It is quite uncertain whether one should distinguish between the slime coat and the plasmalemma proper, and whether proteins are bound in one or another part of this complex structure. Electron microscopy should help to solve this, but the electron micrographs of amoebae that have been published in the past seem rather unsatisfactory from this point of view.⁸

Recently we tried to approach the problem of identifying the binding sites or binding substance on the amoeba's surface. We attempted to make "protoplasts" or naked amoebae by using lysozyme to digest away the slime coat.⁹ The outcome of these experiments was equivocal because, although clouds of material seemed to diffuse from the surface of the amoebae during the treatment in lysozyme, it was not possible to remove completely the PAS-reactive mucoid material, short of lysis or disruption of the amoebae.

Although their outcome was equivocal, these experiments led us to investigate further the effects of lysozyme on *C. chaos*. Chapman-Andresen and Prescott had reported that lysozyme was one of several proteins that induced active pinocytosis.⁴ During our attempts at protoplast formation we found that, when large numbers of amoebae were treated with 0.1 per cent lysozyme at neutral pH, most of the lysozyme disappeared from the supernatant. This suggested that it should be possible to study the binding of lysozyme, and perhaps to isolate the components of the amoebae responsible for binding by working with mass quantities of amoebae.

To obtain the necessary quantities we have maintained mass cultures of *C. chaos* by a procedure that differs only in some details from that described by Prescott.¹⁰ The amoebae are washed and fed daily on a concentrated suspension of washed *Paramecium aurelia*. Every two weeks, about 3 gm. of amoebae are harvested, and the remainder are used to continue the cultures.

Most of our studies have been done with homogenates. The amoebae were washed and starved for 2 to 3 days. They were packed by brief centrifugation at 1000 g, were weighed, and then were homogenized in 0.1 M glycine containing 0.005 M Versene, at pH 6.7. The cells were broken up very easily by drawing them in and out of a bulb pipette. All procedures were done at 0° to 5° C. to avoid gelation and other changes as much as possible.

From such homogenates, we separated by centrifugation at 5000 g a fraction containing the larger particulates, including the mitochondria, the nuclei, and broken sheets or fragments of plasmalemma. By repeated suspension and

centrifugation in the same medium, all the RNA appeared to be removed from the particulate fraction. (We have used the removal of RNA from the particulates as a criterion for judging whether our washing procedure solubilized the remnants of hyaline cytoplasm that must contaminate the membranes at the outset.) This washed particulate fraction was suspended in lysozyme solutions at low ionic strength, at pH 6.7, under the same conditions that had obtained when living amoebae were observed to bind lysozyme. When the particulates were centrifuged down and the supernatants were compared spectrophotometrically with the original lysozyme solution, it was found that much of the protein had been bound. From several simple experiments of this type we found that the particulate fraction derived from 1 gm. of amoeba could bind 12 mg. of lysozyme. This amounts to about 0.5 μ g. per amoeba, roughly one tenth of the total dry weight of the amoeba.

When the particulate fraction containing bound lysozyme was extracted with 0.5 M KCl at neutral pH, all of the lysozyme was extracted, but its solubility appeared to be altered in an interesting manner. If the extract was dialyzed or diluted to an ionic strength of 0.1 or less, almost all of the lysozyme was precipitated. By repeated solution and precipitation we obtained a product that consisted mostly of lysozyme, but that appeared to be a complex of lysozyme with some substance derived from the amoeba particulates. We have not yet identified the amoeba component; until we have done so, we cannot be certain that the product is not simply a modified preparation of lysozyme, yet several lines of evidence suggest that this is not likely. Analysis by absorption studies in the ultraviolet region suggests that the amoeba component is neither protein nor nucleic acid. When the product was studied by Schumaker in the ultracentrifuge, in 0.5 M KCl, the schlieren pattern showed a major component that sedimented at the rate of 2.1 Svedberg units, which is the rate of lysozyme, but the pattern also revealed a minor component that sedimented at a higher rate (FIGURE 3).

At this stage we can only guess, but our guess is that the substance in the washed particulate fraction that binds the basic protein is a mucoid or lipid component of the membranes, acidic in nature, and that this substance is what we have extracted and purified as the lysozyme complex. Whether the enzymatic activity of lysozyme has anything to do with the process is uncertain. Some evidence suggests that enzymatic activity is not involved, just as the enzymatic activity of ribonuclease appeared to have nothing to do with binding in Schumaker's experiments, but this must be investigated further.

We have made some observations on the supernatant fraction of amoeba homogenates. This fraction contains, roughly, 10 to 15 mg. of protein and about 2 mg. of RNA, per gram of wet amoebae. When this fraction is dialyzed against 0.001 M CaCl_2 , a precipitate of nucleoprotein forms, and from this nucleoprotein one can isolate by gentle procedures a solution of RNA. FIGURE 4 shows the ultraviolet absorption spectrum of such a preparation. Judging by its absorption at the lower wave lengths, it seems to contain little protein. Ribonuclease treatment of this preparation increased the optical density at 260 $\text{m}\mu$ g. by 33 per cent, as shown in the upper curve. Desoxyribonuclease had no effect.

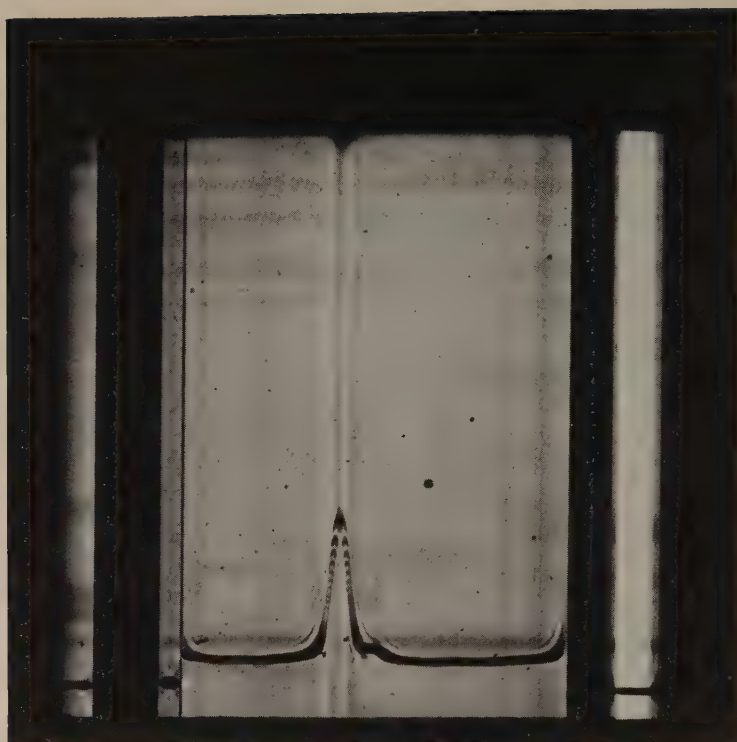


FIGURE 3. Schlieren pattern in ultracentrifuge of lysozyme complex extracted from amoeba particulate fraction. See comment in text.

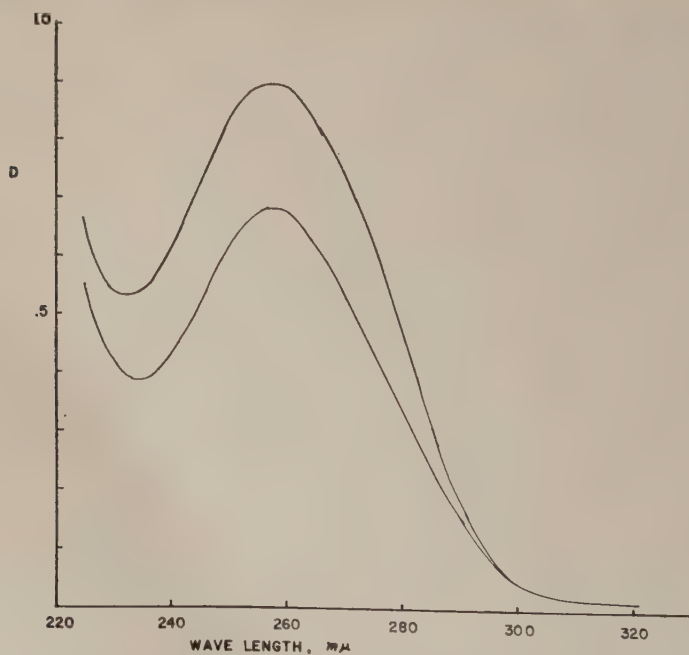


FIGURE 4. Absorption spectrum of RNA preparation obtained from *C. chaos*. Lower curve: before RNase. Upper curve: after RNase digestion.

Lysozyme reacts with the purified RNA preparation to give a typical nucleoprotein precipitate. Lysozyme reacts similarly with the crude homogenate supernatant to precipitate the RNA, apparently by displacing most of the amoeba protein. The lysozyme-RNA complex formed in this way has some interesting properties, but I shall not go into this matter because it does not seem very likely, from our experience with lysozyme binding by intact amoebae, that RNA is an exposed component of the surface, or that it plays any role in the initial binding steps of pinocytosis. However, we are very much interested in the RNA and protein of the cytoplasm as elements in the sol-gel changes¹¹ that Landau discusses in his paper in this monograph, because these changes may control the third stage of pinocytosis, the actual process of engulfment.

Mass cultures of amoebae promised to be useful for studying the hyaline cytoplasm, because one can work them up not only by homogenate techniques, but by even simpler methods that avoid some of the artifacts of homogenization. An example is the following experiment:

Three grams of *C. chaos* were washed in deionized water at 5° C. and were packed by brief centrifugation in the cold at 1300 g. The water was drawn off, leaving a semisolid mass of amoebae with a very small amount of interstitial water. When this mass was centrifuged at 25,000 g for 15 min. at 2° C., there appeared a clear supernatant layer of what I shall call "expressed plasmasol." Analysis by spectrophotometry indicated that the composition was about the

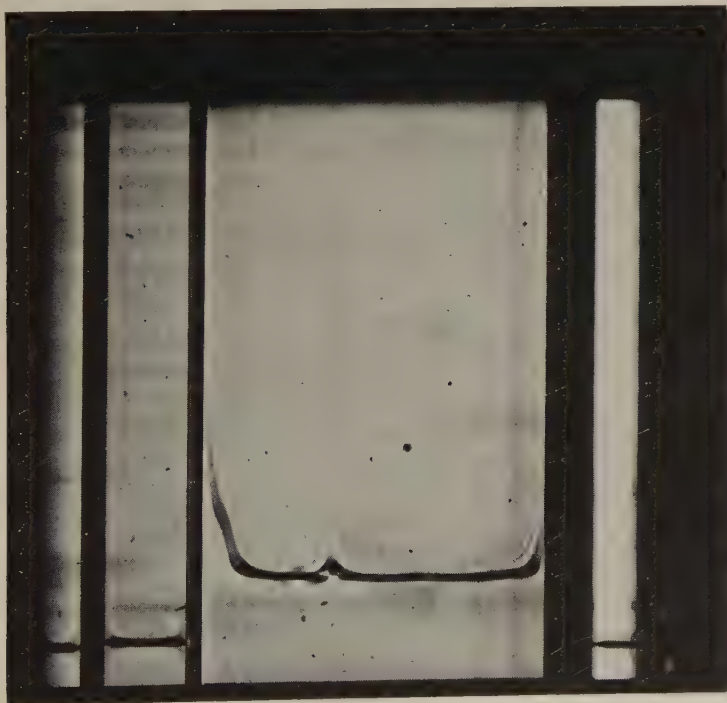


FIGURE 5. Schlieren pattern in ultracentrifuge of expressed plasmasol from *C. chaos*. See comment in text.

same as that of the more dilute supernatant solution obtained from homogenates.

This expressed plasmasol was transferred quickly to the ultracentrifuge and studied at 8° C. by Schumaker. FIGURE 5 shows the schlieren pattern observed early in the run. First, there is a fast-moving component that sediments at the rate of 67 Svedberg units. A photograph taken by the ultraviolet optical system during this run suggests that a good deal of the nucleic acid in the expressed plasmasol may be carried down with this rapidly sedimenting component. The sedimentation coefficients given here were not corrected to infinite dilution, but the large correction to 20° C. was made. These observations recall to mind the sedimentation characteristics of the nucleoprotein particles, or microsomes, from yeast, studied by Chao and Schachman,¹² and from rat liver, studied by Petermann and Hamilton.¹³ Both yeast and liver microsomes show sedimentation constants, extrapolated to infinite dilution, of about 80 Svedberg units.

Later in the run (FIGURE 6) the schlieren pattern shows the beginning of the formation of the major peak. This peak probably contains most of the protein of the expressed plasmasol, and it sediments at the rate of 3.9 Svedberg units. There is a shoulder on the major peak that sediments at about 21 S. These values also are uncorrected for concentration dependence.

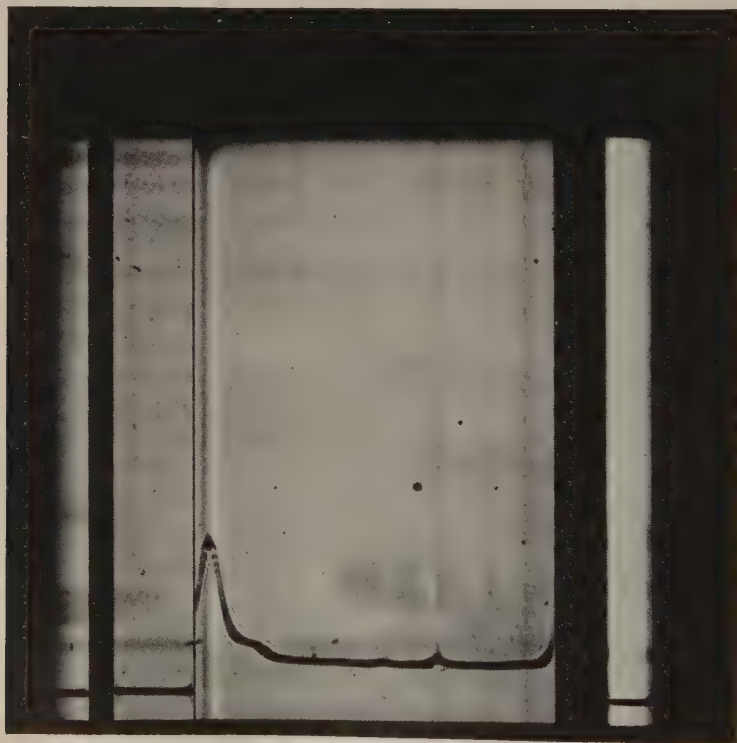


FIGURE 6. Later photograph from same ultracentrifuge run as in figure 5. See comment in text.

Summary

A start has been made at analyzing experimentally the mechanisms of pinocytosis in amoebae.

Protein uptake by pinocytosis proceeds in definite stages. Schumaker's work defined some of the characteristics of each stage, and Brandt's cytological study led to similar conclusions.

As a working hypothesis, it is suggested that pinocytosis begins with the binding of proteins or other effective inducers to the cell surface, the essential groups of the bound substance being positively charged, those on the cell surface being negative. Several characteristics suggest that the binding is salt-like.

Some experiments with the binding of lysozyme by fractions prepared from homogenates of *C. chaos* support this hypothesis. Work in progress suggests that it may be possible to isolate and identify chemically the components of the cell surface that bind lysozyme.

Other observations on homogenates indicate that it is possible to isolate RNA from amoebae by gentle procedures with relatively little degradation.

Some examples have been given of ways in which mass quantities of *C. chaos* may be used to study the physical and chemical properties of the cytoplasm.

Acknowledgment

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References

1. LEWIS, W. H. 1931. Pinocytosis. *Bull. Johns Hopkins Hosp.* **49**: 17-27.
2. MAST, S. O. & W. L. DOYLE. 1934. Ingestion of fluid by amoeba. *Protoplasma*. **20**: 555-560.
3. HOLTER, H. & J. M. MARSHALL, JR. 1954. Studies on pinocytosis in the amoeba *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **29**: 7-27.
4. CHAPMAN-ANDRESEN, C. & D. M. PRESCOTT. 1956. Studies on pinocytosis in the amoebae *Chaos chaos* and *Amoeba proteus*. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **30**: 57-78.
5. SCHUMAKER, V. N. 1958. Uptake of protein from solution by the *Amoeba proteus*. *Exptl. Cell Research*. **15**: 314-331.
6. BRANDT, P. W. 1958. A study of the mechanism of pinocytosis. *Exptl. Cell Research*. **15**: 300-313.
7. BAIRATI, A. & F. E. LEHMANN. 1953. Structural and chemical properties of the plasmalemma of *Amoeba proteus*. *Exptl. Cell Research*. **5**: 220-233.
8. LEHMANN, F. E., E. MANNI & A. BAIRATI. 1956. Der Feinbau von Plasmalemma und kontraktile Vakuole bei *Amoeba proteus* in Schnitt und Fragmentpräparaten. *Rév. suisse zool.* **63**: 246-255.
9. WEIBULL, C. 1956. Bacterial protoplasts; their formation and characteristics. In *Bacterial Anatomy*. : 111-126. E. T. C. Spooner & B. A. D. Stocker, Eds. Cambridge Univ. Press. Cambridge, England.
10. PRESCOTT, D. M. 1956. Mass and clone culturing of *Amoeba proteus* and *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **30**: 1-12.
11. MARSLAND, D. 1956. Protoplasmic contractility in relation to gel structure: temperature-pressure experiments on cytokinesis and amoeboid movement. *Intern. Rev. Cytol.* **5**: 199-227.
12. CHAO, F.-C. & H. K. SCHACHMAN. 1956. The isolation and characterization of a macromolecular ribonucleoprotein from yeast. *Arch. Biochem. Biophys.* **61**: 220-230.
13. PETERMANN, M. L. & M. G. HAMILTON. 1957. The purification and properties of cytoplasmic ribonucleoprotein from rat liver. *J. Biol. Chem.* **224**: 725-736.

PROBLEMS OF PINOCYTOSIS, WITH SPECIAL REGARD TO AMOEBAE

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The fascinating work that Marshall reviews in this monograph has introduced the reader to some of the most recent advances in the study of pinocytosis. It is my purpose to report some of the studies that have been carried out at the Carlsberg Laboratory, and also to relate some results obtained in other laboratories. Although several of these results have been gained by means of organisms other than amoebae, it was necessary to include them because they are of the greatest significance for the general problem of pinocytosis.

I feel that I cannot begin my paper in a more fitting way than by paying respect to the pioneers in the field, Warren Lewis, S. O. Mast, and W. L. Doyle, who twenty-five years ago discovered and analyzed the process of pinocytosis in mammalian tissue culture cells and in amoebae. The more I see of pinocytosis, the more I admire the accuracy of these scientists' early observations, obtained without phase contrast and without labeled tracers. The discoveries of these workers passed almost unnoticed for a long time. This sounds amazing, but the reason I think, is fairly simple: pinocytosis is difficult to study without techniques that have been developed in recent years.

The first problem I propose to discuss is whether we are right in thinking of pinocytosis primarily as an ingestion of fluid. The very word pinocytosis, as coined by Lewis, is derived from *πινειν*, which means to drink, and it certainly emphasizes this side of the concept. However, it is becoming more and more clear that the substances that are dissolved in the fluid are even more crucial for pinocytosis than the fluid itself. In order to discuss this question properly, I must first say a few words about the morphology of pinocytosis.

In tissue culture cells, as may be seen in the time-lapse films made by Lewis¹ himself and later by Frederic and Chèvremont,² Gey,³ and Pomerat⁴ and their co-workers, and most recently by John Paul⁵ pinocytosis is entirely dependent on the presence of membranous ruffle pseudopodia. By their undulating movements these membranes enclose droplets of the fluid that surrounds the cells, thus forming vacuoles. The average diameter of the primary pinocytosis vacuole in macrophages is about 1 to 2 μ ; their shape is initially dependent on the shape of the membrane cavity, but as they enter the interior of the cell they soon become spherical. This type of pinocytosis is shown by a great number of cells. In addition to those usually grown in tissue culture, I shall only mention leukocytes and ascites tumor cells, which my co-worker, C. Chapman-Andresen is studying at present.

The morphology of pinocytosis is somewhat different in the case of amoebae. The essential feature in these organisms is not the undulating function of a membranous pseudopod, but the formation of a tubelike channel from which the pinocytosis vacuoles are pinched off. This mechanism was seen and de-

scribed by Mast and Doyle⁶ and, in our later studies, we have been able to confirm their observations in all essential features (FIGURES 1 and 2). Much has already been said in this monograph about the details and the mechanism of channel formation, and I do not propose to repeat any of it. However, I must emphasize that the pinocytosis channel of the amoebae, although characteristic for these organisms, is a variable feature morphologically. At the

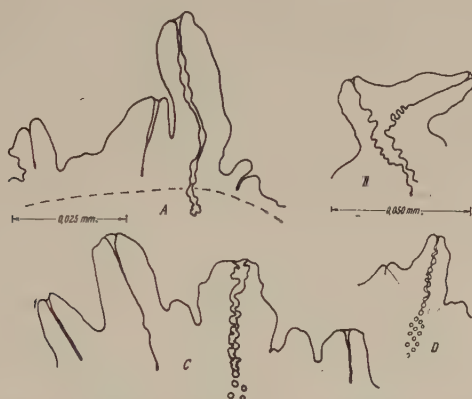


FIGURE 1. Pinocytosis in amoebae, as depicted by Mast and Doyle. After Mast and Doyle.⁶

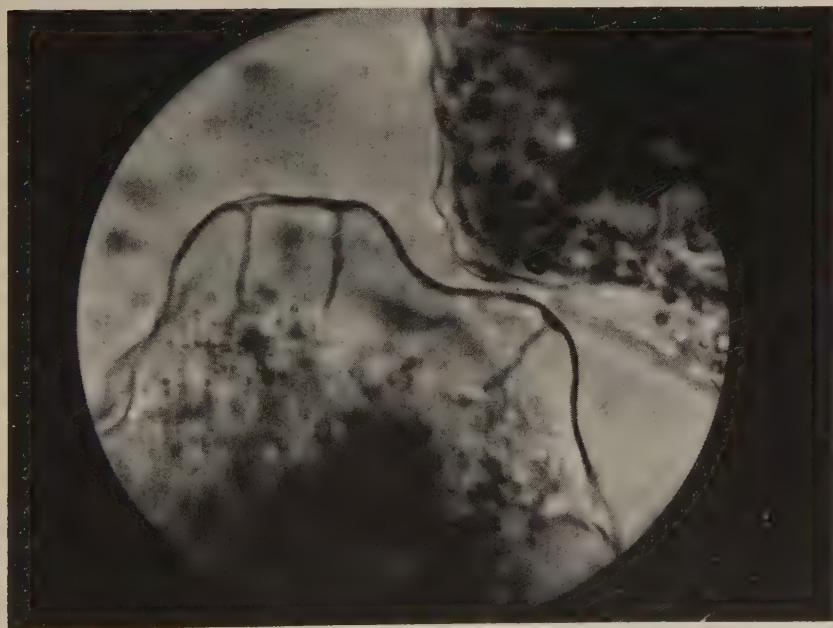


FIGURE 2. Pinocytosis channels in *Amoeba proteus*: 1.5 per cent bovine plasma albumin. $\times 850$. After Chapman-Andresen and Prescott.⁷

Carlsberg Laboratory, Chapman-Andresen and Prescott⁷ have described a continuous series of channel types, from food-cuplike cavities, strongly reminiscent of phagocytosis, to very narrow tubes yielding pinocytosis vacuoles of $1\ \mu$ diameter or less (FIGURE 3).

In later investigations on the morphology of pinocytosis, Chapman-Andresen⁹ has obtained additional evidence that the morphology of channel formation is at least partially dependent on the nature of the fluid that is ingested. I shall not go into details of her findings; I shall mention only the fact that different solutes seem to induce different types of channels, a finding that agrees well, of course, with the assumption that the first stage of pinocytosis is the interaction of the solute with the surface of the amoeba, the phenomenon already discussed in this publication.

The instances of pinocytosis that I have mentioned thus far have been within the dimensions of microscopic visibility. Recently, however, an extremely interesting extension of the pinocytosis concept has emerged from work with the electron microscope. This is due mainly to observations made by Palade,¹⁰ which have led Stanley Bennett to formulate his hypothesis of membrane flow and membrane vesiculation¹¹ (FIGURE 4).

Bennett's hypothesis postulates that particles, molecules or ions, are in some way engaged by the cell surface membrane (FIGURE 4A, A') are included in a vesicle pinched off from the tip of the recess and may thence be moved to some other portion of the cell (FIGURE 4B, left). In addition, Bennett proposes a mechanism by means of which vesicles are formed directly at the surface membrane. This mechanism consists again in the engagement of particles at the cell surface by appropriate forces, followed by an invagination of the surface at the loaded area and a pinching off of the invaginated part. In both cases Bennett then assumes an enzymatic breakdown of the isolated membrane, leading to a liberation of enclosed substances (FIGURE 4a to f, right).

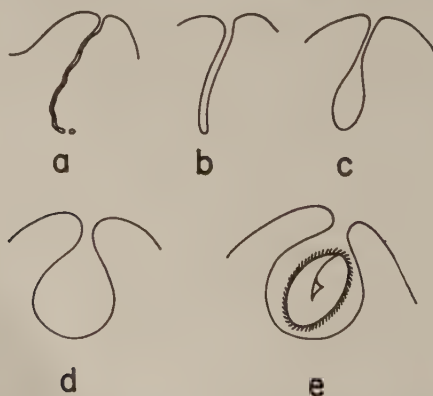


FIGURE 3. Diagrams of different microscopically visible modes of ingestion in amoebae: (a) typical pinocytosis channel in salt solution; (b) pinocytosis channel in solution of tobacco mosaic virus; (c) "bottle-shaped" cavity in solution of tobacco mosaic virus; (d) cavity formed in optically clear methionine solution; and (e) formation of food vacuole containing small ciliate. After Chapman-Andresen and Prescott.⁷

It is obvious that these hypothetical mechanisms very closely resemble what can be actually seen in amoebae, only on a much larger scale than the one with which Palade's observations and Bennett's hypothesis are mainly concerned. Bennett, of course, was quite aware of the close connection between membrane vesiculation and pinocytosis, and Palade uses the expression "pinocytosis at the submicroscopic level."

The work of Brandt and Schumaker, mentioned elsewhere in these pages by Marshall, constitutes a very remarkable support of Bennett's hypothesis. I may add that Chapman-Andresen and Betty Danes at the Carlsberg Laboratory, working with radioactive sodium chloride and radioactive gold, have obtained results that can perhaps best be interpreted on the assumption that in this case, too, surface adsorption comes into the picture.

Insofar as I can see, therefore, there is considerable evidence in favor of Bennett's proposal, and there is scarcely any reason why one should not accept it. If we do so, and regard membrane vesiculation as an extension of the visible pinocytosis phenomenon toward molecular dimensions, we arrive at a preliminary definition of pinocytosis as a mechanism for the discontinuous uptake of fluids by invagination and vesiculation of the cell surface. The quantities taken up at a single gulp cover a wide range, and there is no sharp demarcation against phagocytosis on the one side and molecular permeation on the other.

I now propose to discuss the more physiological side of the question of how pinocytosis is induced. In the definition just given it was stated that pinocytosis is a discontinuous process. This is true in two senses: (1) the fluid is taken up not in a continuous stream, but in sips, or gulps, the size of which is determined by the size of the primary pinocytosis vacuoles (as we have seen this size is extremely variable); and (2) the process as such is discontinuous in the sense that the cells are not drinking all the time. In tissue cultures it is known—and has already been observed by Lewis—that pinocytosis is not going on continuously, but nothing is known about what makes the process start or stop. In amoebae, the process can be initiated at will by the addition of certain substances, and this, to my mind, is perhaps the greatest advantage that

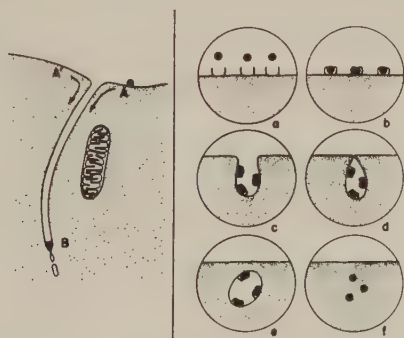


FIGURE 4. Left: diagram representing transport by membrane flow of a particle from surface sites (*A* and *A'*) to the interior of the cell (*B*); right: diagram representing successive stages (*a* to *f*) of membrane vesiculation. After Bennett.¹¹

amoebae offer in the study of pinocytosis. Working with these organisms, we have a chance of finding out which molecular properties are responsible for the induction or, in other words, what kinds of substances are bound by the binding sites that Bennett's hypothesis presupposes. At our laboratory we have investigated this question for some time, but thus far without much success. Broadly speaking, it can be said that the most efficient inducers of pinocytosis known at present are salts and proteins. Within these groups, however, there are specific differences. Chapman-Andresen⁹ has studied the induction of pinocytosis in *Amoeba proteus* by various simple inorganic salts, and she has found that different cations, such as sodium and potassium, induce slightly different morphologic types of pinocytosis with regard to the width of the channels and the way they are formed. She has also found that there exists, for every salt, an optimum range of concentration where pinocytosis is intense and where toxic or osmotic effects are still not sufficiently serious to prevent the inducing effect. The optimum range also shows specific differences but, with most salts investigated, is in the neighborhood of one-tenth molar. Most of these effects were not very well defined, and strictly quantitative data are still lacking, but one thing is certain, namely that there is no simple correlation between the osmotic conditions offered by the various solutions and their pinocytosis-inducing efficiency.

The same thing is true with regard to proteins and a number of other substances tested in our laboratory, mainly by Chapman-Andresen and Prescott.⁷ Here again there are specific differences, remarkable especially within the protein group, but so far no simple correlation between chemical properties and inducing power has been found. The best inducers in our experience, in addition to the inorganic salts, were rabbit gamma-globulin, gelatin, and Na-glutamate. Noninducing are: all carbohydrates tested, whether low- or high-molecular; also, surprisingly enough, the nucleic acids. All we can honestly say at present is that for unknown reasons some substances seem to taste better than others.

However, we do not intend to dismiss this question of specificity simply as a matter of taste. It may be the key to our understanding of the surface adsorption that initiates the process of pinocytosis, and we plan to investigate it further, but I fear that it will be a long and tedious task.

Another problem for discussion is the measurement of the amounts of fluid actually taken up by pinocytosis. The first investigation of this question was done by Marshall and myself.⁸ We fed *Chaos chaos* with fluorescein-labeled rabbit gamma-globulin. By means of a micromethod devised by Marshall, the amount of fluorescent material present in an amoeba was determined and the corresponding uptake of protein solution was calculated on the assumption that the amounts of fluid and of protein would be proportional (TABLE 1).

In this way it was found that an amoeba with gamma-globulin as an inducer pinocytoses about 30 per cent of its own volume in the course of 3 hours. Lewis, by judging the diameter and number of the vacuoles in his macrophages, arrived at a similar order of magnitude in 1931¹ and, in a later paper,¹³ he states that the cells may take up several times their own volume within a few hours.

TABLE 1*

VOLUMES OF PROTEIN SOLUTION PINOCYTOSED, AS CALCULATED FROM AMOUNT OF
FLUORESCENT PROTEIN TAKEN UP AND DETERMINED IN MICROCUVETTE

No.	Volume in cuvette, μ l.	I ₅₂₅	I ₄₃₀	ΔI_{525}	Volume of solu- tion ingested, μ l.
1.....	0.69	32.5	12.3	16	7.2
2.....	0.69	49.0	17.8	25	11.3
3.....	0.69	40.5	16.3	18.5	8.4
4.....	0.60	50.0	20.5	22.5	8.8
5.....	0.57	53.5	22.3	23.5	8.8
6.....	0.60	51.5	19.5	25.5	10.0
7.....	0.64	46.0	15.3	25.5	10.7
8.....	0.61	48.0	19.0	22.5	9.0
9.....	0.60	48.5	17.5	25	9.8
10.....	0.60	53.5	18.5	28.5	11.2
Mean					9.5 \pm 0.4

Calculations:

$I_{525, \text{protein}} = 1530 \text{ scale units, on this day.}$

$\Delta I_{525} = I_{525} - I_{430} \times 1.34$

Volume ingested = $\frac{\Delta I_{525}}{1530} \times (\text{volume in cuvette})$

* Reproduced by permission from Holter and Marshall.⁸

According to all this evidence, therefore, the volumes of fluid taken up seemed to be rather remarkable.

Considering what we know today, Marshall's and my data still hold good as regards protein uptake, but the deductions from these data as to volume of fluid ingested are no longer valid. If there occurs an adsorptive concentration of protein prior to ingestion, the protein uptake cannot be taken as a true measure of the fluid ingested. For future investigations of this type, a double label will be necessary and, until such experiments have been carried out, we shall not know much more about the fluid volume than Lewis could deduce from his time-lapse pictures. It is comforting to know that these rough estimates lead to orders of magnitude similar to those given by the labeling experiments.

However, there exists already one series of measurements that may be regarded as an approximation to a double label experiment. In order to introduce radioactive glucose into amoebae, Chapman-Andresen and I¹⁴ used protein as an inducer of pinocytosis, unfortunately not gamma-globulin, as in Marshall's experiments, but bovine plasma albumin. Glucose itself does not induce pinocytosis; it is therefore presumably not adsorbed to the plasmalemma, and the amounts of glucose taken up may be assumed to represent a true measure of the actual volume of fluid ingested (TABLE 2).

If we compare the results shown in this table with the fluid volumes calculated from the uptake of labeled gamma-globulin shown in TABLE 2, two main differences are to be noted. One is that the ingestion of fluid seems to be much more capricious. Whereas in the previous experiment all amoebae ingested

TABLE 2*
 UPTAKE OF C¹⁴ GLUCOSE BY *Chaos chaos*

Group	Feeding solution	Total No. of amoebae	No. of amoebae ingesting activity corresponding to following volumes of active solution		
			>4 μl.	between	<0.4 μl.
I	Pringsheim-glucose	50	0 (0%)	3 (6%)	47 (94%)
II	Pringsheim-glucose with lycopodium spores	12	0 (0%)	4 (33%)	8 (67%)
III	Protein-glucose	109	12 (11%)	54 (50%)	43 (39%)
IV	Protein-glucose new batch protein, prewash in protein	116	16 (14%)	72 (62%)	28 (24%)

* Reproduced by permission from Chapman-Andresen and Holter.¹⁴

roughly equal amounts of protein with surprising regularity, the fluid uptake in this case, measured as the ingestion of glucose, shows individual variations ranging over more than a power of ten. The other difference is that, in the case of albumin-glucose, only few amoebae took up fluid in the order of 10 per cent or more of their own volume, while the amount calculated from the gamma-globulin uptake seemed to be about 30 per cent.

At the time these results were obtained we regarded them simply as an indication that bovine plasma albumin was a less efficient inducer of pinocytosis than gamma-globulin. However, in the light of our present knowledge it seems quite possible that the differences may be due to the fact that, with glucose as a nonconcentrating tracer, we have obtained a measure simply of the actual amounts of fluid ingested while, in the case of labeled gamma-globulin, we were measuring the sum of the protein in solution in the vacuoles plus that concentrated on the membranes.

If this suspicion should turn out to be correct, one would have to deduce that ingestion volume is a much more irregular and unreliable characteristic of pinocytosis than ingestion area—a result that seems at first sight somewhat surprising in view of the original definition of pinocytosis. However, considering Brandt's and Palade's results and Bennett's hypothesis, it seems by no means inconceivable. As I said, experiments with a double label are desirable in order to explore this problem further.

The next point I propose to discuss is the fate of the pinocytosis vacuoles after ingestion. The first thing that can be observed is a rather intense process of dehydration. In tissue culture cells, where the vacuoles are comparatively stationary and can be followed individually in time-lapse films, the shrinkage of the vacuoles can be seen directly. In amoebae, where the cytoplasmic currents prevent direct observation, the situation is more complicated. Measurements of average vacuole diameters at different times after ingestion are useless, since both division and coalescence of primary pinocytosis vacuoles have actually been observed and probably occur quite frequently. However, the shrink-

age of vacuoles and the corresponding concentration of their contents can be demonstrated by centrifugation. At the moment of ingestion, and even assuming a considerable concentration of protein by surface adsorption, the vacuoles must be lighter than the average density of the amoeba cytoplasm. A few hours later the vacuoles, by centrifugation of the living amoeba, can be accumulated in a stratum rather close to the heaviest components of the cytoplasm (FIGURE 5). The shrinking process continues and, finally, when the vacuoles have been reduced to the size of granules, they accumulate upon centrifugation in the same zone as the mitochondria (FIGURE 6).



FIGURE 5. Heavy pole of a *Chaos chaos*, centrifuged and fixed 2 hours after feeding. Fluorescent protein vacuoles occur throughout the stratum containing mitochondria and nuclei. $\times 600$. After Holter and Marshall.⁸



FIGURE 6. Heavy pole of a *Chaos chaos*, centrifuged and fixed 3 days after feeding. Mitochondrial-like fluorescent granules. $\times 600$. After Holter and Marshall.⁸

This brings us to another one of the problems of pinocytosis: namely, the supposed connection between pinocytosis vacuoles and mitochondria. Gey *et al.*³ have claimed that mitochondria are formed by the fusion of shrunken pinocytosis granules with pre-existing mitochondria, or are produced *de novo* by the formation of chains of pinocytosis granules. In his beautiful pictures Brandt has shown that the pinocytosis vacuoles contain many infoldings of the vacuolar membrane, and one cannot help being struck by the morphologic similarity of these structures and the cristae that are such an essential feature of the electron microscopic pictures of mitochondria. Of course, this resemblance may indicate only a case of morphologic convergence, due to the membranous origin of both structures. However, it may also indicate a closer connection. Considering what we know today about the ultrastructure of mitochondria and their biochemical properties, it is certainly difficult to imagine a transition from a simple vacuole to a mitochondrion; however, if the essential feature of this vacuole is a highly developed internal surface with the characteristics of the plasmalemma, such a transition becomes perhaps less improbable, also with regard to enzymatic properties. This is an exceedingly interesting problem and a challenging task for cytochemists and electron microscopists.

Another main problem of pinocytosis of which, thus far, we know very little, is the following: what happens to the solutes during the transformation of the pinocytosis vacuole to a cytoplasmic granule? Lewis¹ calls this transformation "digestion," but I am afraid that this is perhaps a little bit oversimplified. After all, one of the most interesting physiological aspects of pinocytosis is that it may enable cells to take up and incorporate, as such, high molecular substances that they cannot take up by diffusion. However, this hypothesis must be abandoned if one assumes, by the word digestion, an enzymatic break down of large molecules into smaller ones, in order to enable them to permeate the vacuolar membrane. This is why the question of the permeability of the vacuolar wall becomes so interesting, and we have therefore begun to study it in amoebae.

As I have said previously, the surface of *Chaos chaos* is very impermeable. For instance, amoebae of this species do not take up glucose to any appreciable extent, and even their permeability to water is very low.¹⁵ Pinocytosis takes place by an invagination of this very impermeable surface, and the vacuole formed is enclosed by a membrane that originally was a part of the cell membrane. There is no obvious reason for assuming, and no microscopic evidence to indicate, that the permeability of this membrane should be changed during the act of invagination but, as long as it is unchanged, the contents of the vacuole must be assumed to be just as inaccessible to the organism as if they were still in the surrounding medium.

In order to find out by means of a simple model if and how soon the vacuolar membrane becomes more permeable, Chapman-Andresen and I fed the amoebae C¹⁴-labeled glucose and followed its distribution by means of an autoradiographic technique.¹⁴ The glucose was dissolved in a plasma albumin solution as a vehicle for pinocytosis. I shall not say much about the technique used; I merely point out that glucose is not a very pleasant substance with

TABLE 3*
RECOVERY OF ACTIVITY FROM AMOEBAE AFTER FREEZE-DRYING,
PARAFFIN-EMBEDDING, AND SECTIONING

Amoeba No.	Activity <i>in vivo</i> Cpm	Cpm/ sectioned amoeba	Percentage recovery
P.G. 10,4	271	278	102.6 \pm 4.3
P.G. 10,14	169	183	108.3 \pm 4.2
P.G. 10,17	86	87	101.2 \pm 4.3
P.G. 15,7	114	114	100.0 \pm 4.3
P.G. 8,3	54	52	96.3 \pm 4.4
P.G. 7,5	99	100	101.0 \pm 4.1

* Reproduced by permission from Chapman-Andresen and Holter.¹⁴

which to work, since it is water-soluble and cannot be fixed. The activity of the amoebae was first measured *in vivo* in a compression chamber; then they were freeze-dried, sectioned, and mounted dry without removal of paraffin. The recovery is shown in TABLE 3.

For radioautography, the amoeba sections were covered by 0.25 μ nylon film in order to protect them against water during the application of the stripping-film emulsion.^{16, 17} One of the autoradiograms obtained is shown in FIGURE 7.

The autoradiogram shows that there is a considerable amount of activity evenly distributed in the cytoplasm of the amoebae. Such autoradiograms have been obtained as early as 45 min. after the end of feeding. It is therefore

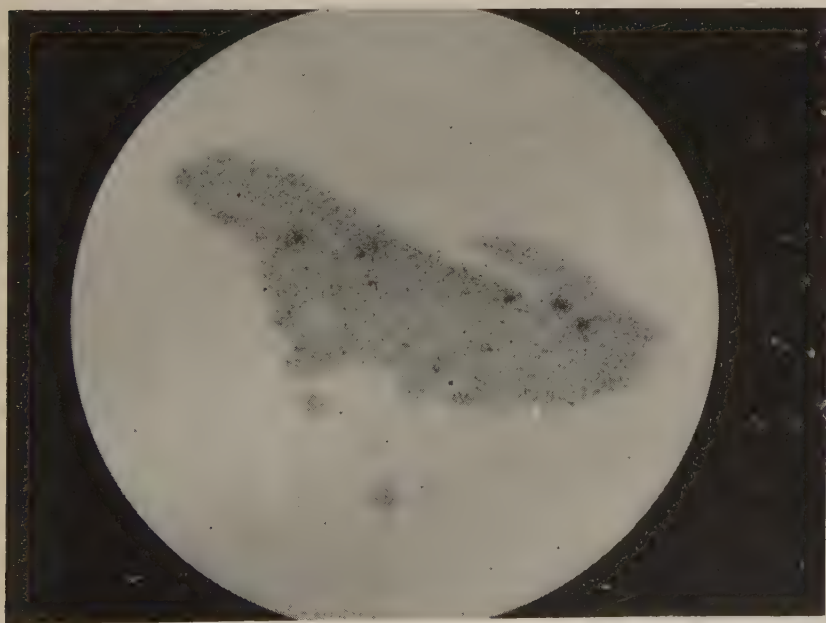


FIGURE 7. Autoradiogram of 10 μ section of *Chaos chaos* freeze-dried 3½ hours after uptake of glucose-protein solution. $\times 500$. After Chapman-Andresen and Holter.¹⁴

reasonable to assume that the process of glucose resorption begins soon after the formation of the vacuoles; in other words, the impermeability of the cell surface is not maintained by the vacuolar membrane. Whether the mechanism of penetration is a diffusion of unchanged glucose or an active resorption by means of enzymatic equipment newly formed at the membrane is not known.

By transferring the amoeba to a Cartesian diver and following its respiration it can be shown that the radioactive glucose taken up by pinocytosis enters into the normal metabolism of the amoeba. Most of it is recovered in the respiratory carbon dioxide, about 10 to 20 per cent is incorporated in a more permanent way in the cytoskeleton.

The cytoplasmic localization of this remaining activity was studied by means of radioautographs of centrifuged amoebae (FIGURE 8). FIGURE 8 shows such an autoradiogram of an amoeba freeze-dried 123 hours after the uptake of glucose. The highest concentration of activity corresponds to the layer in which the mitochondria are accumulated. However, this is probably just an expression of the generally high metabolic activity of mitochondria, and it may have no specific relation to pinocytosis as such. This is demonstrated in FIGURE 9.

This figure shows the quantitative distribution of radioactivity in the same amoeba, as determined by counting and plotting the number of silver grains per unit area along the main axis of the amoeba. The upper diagram shows the activity of a centrifuged amoeba that had been fed with a C^{14} -marked ciliate by way of ordinary phagocytosis. The curve shows the same concentration

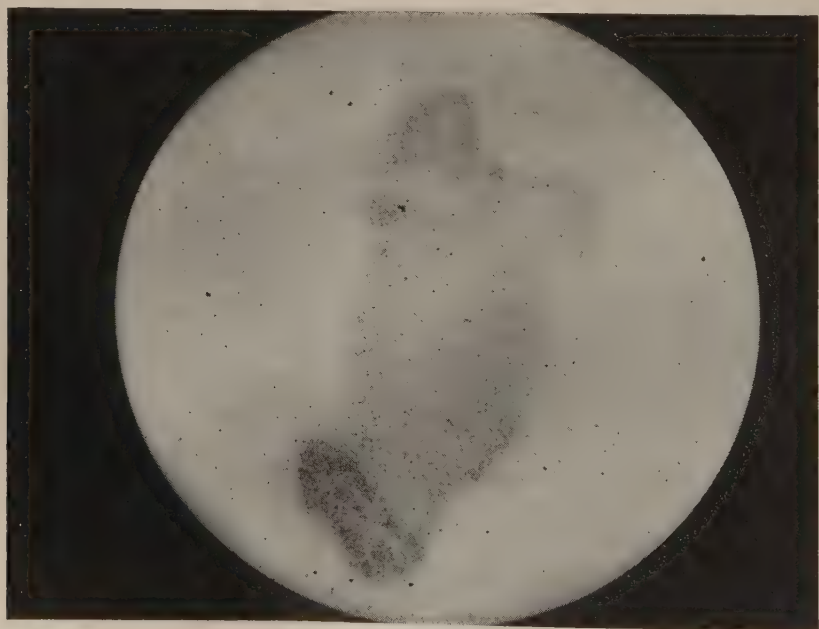


FIGURE 8. Autoradiogram of $10\ \mu$ section of *Chaos chaos* centrifuged and freeze-dried 123 hours after uptake of glucose-protein solution. $\times 500$. After Chapman-Andresen and Holter.¹⁴

of activity in the mitochondrial region and, in addition, another concentration of activity in a lighter zone, corresponding to a nongranular layer of hyaline cytoplasm presumably rich in lipids. This band has not been observed in amoebae fed with glucose.

Results similar to the glucose findings were obtained by Chapman-Andresen and Prescott⁷ with regard to the incorporation of an amino acid, methionine. There can therefore not be much doubt that at least low-molecular food substances, given by pinocytosis, are being utilized.

About the fate of high-molecular substances, especially proteins, we know as yet very little. After ingestion of fluorescein-labeled protein, the label is eliminated completely in 5 to 6 days, in contradistinction to radioactive carbon from glucose, a significant amount of which is retained (FIGURE 10).

We have no means of ascertaining directly whether curve *C* depicts the digestion of the marked protein within the pinocytosis vacuoles, or outside the vacuoles in the cytoplasm, or whether it depicts simply the subsequent process of elimination of the unphysiological labeling substance.

We had hoped to obtain additional information by studying the uptake of radioactive protein by pinocytosis, but thus far we have not been able to obtain soluble proteins that were sufficiently radioactive and suitable for autoradiography. However, even if we should succeed in carrying out such experiments, it must be realized that they probably can give information only about the integrated nutritive utilization of such proteins. Autoradiography can hardly solve the question: by what mechanism and in what state are the protein molecules being transferred from the vacuoles to the cytoplasm? For this, some other experimental approach must be found.

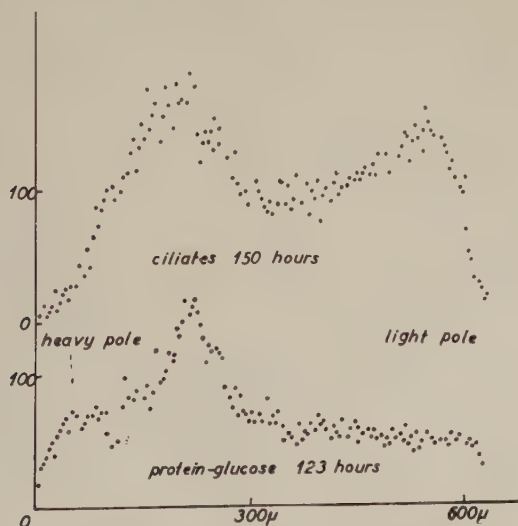


FIGURE 9. Silver grain counts on autoradiograms of $10\ \mu$ sections of centrifuged freeze-dried amoebae. Upper curve: amoeba fixed 150 hours after uptake of active ciliates by phagocytosis. Lower curve: amoeba fixed 123 hours after uptake of glucose-protein solution by pinocytosis (same amoeba as in FIGURE 8). After Chapman-Andresen and Holter.¹⁴

The question is of crucial importance for the evaluation of the physiological role of pinocytosis. If pinocytosis is to be considered as a mechanism for the uptake of high-molecular substances without breakup of the molecules, we must be able to answer the question of how the membrane barrier is overcome.

It has been supposed that the vacuolar membrane itself is digested by cytoplasmic enzymes, so that the contents of the vacuoles are released into the cytoplasm. This supposition would involve some rather interesting assumptions about the instability of a cytoplasmic structure against the cell's own enzymes, a kind of local cytolysis, so to speak, but thus far no evidence of this has been found. The membrane is certainly not rigid, since the vacuoles can be deformed, can shrink and can both coalesce and divide, but we have never seen it disappear. Further evidence on this point is thus highly desirable.

However this may be, the fact that at least low molecular substances do penetrate, enables us, by means of pinocytosis, to study the metabolism of substances that would not be taken up normally. I have already mentioned the fact that an amoeba can be persuaded to ingest and utilize carbohydrate, if it is offered together with a pinocytosis-inducing protein. Ribonuclease, the effects of which have been studied in Brachet's laboratory, also enters the amoeba by pinocytosis. I cannot say whether a similar situation exists in the case of tissue culture cells with their more constant environment. I know of one instance, demonstrated in a film by John Paul,⁵ where the addition of insulin to the medium brought about a very impressive intensification of pinocytosis. I believe strongly that a systematic investigation of the nutritional side of pinocytosis would be rewarding.

Finally I propose barely to mention another big problem of pinocytosis, the transport of the vesicles from the surface into the cell. In tissue culture cells,

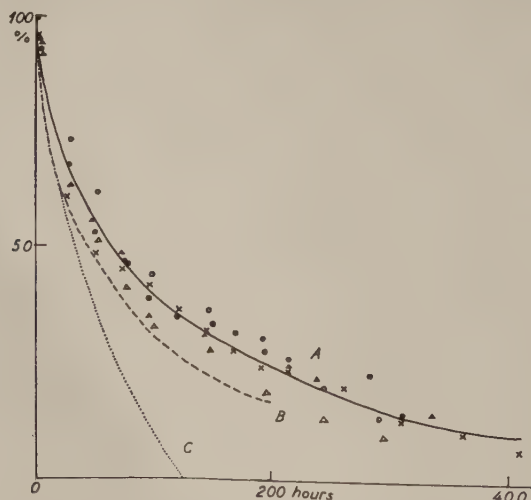


FIGURE 10. Disappearance of radioactivity from *Chaos chaos*. (A) After ingestion of C^{14} glucose by pinocytosis; (B) after ingestion of radioactive ciliates; (C) excretion of fluorescent label after pinocytosis of fluorescent protein. Symbols to A indicate repeated measurements on 5 individual amoebae. After Chapman-Andresen and Holter.¹⁴

where every vacuole can be followed on its way from the periphery to the perinuclear space, this process is very impressive. It has already occupied Warren Lewis,¹ and the explanation of its mechanism by membrane flow is a very essential feature of Bennett's¹¹ hypothesis. I am afraid that studies on amoebae can contribute very little to this problem because of the cytoplasmic currents that are characteristic of these organisms.

Concluding Remarks

Throughout this paper it has been my primary intent to convey to you the feeling that pinocytosis is one of the fundamental processes in the physiology of amoeboid cells. It is true, in a sense, that the generalized picture of pinocytosis that can be given today is more confusing than it seemed a few years ago. Indeed, the original definition of pinocytosis is today in doubt, and it may require modification toward less emphasis on the fluid uptake and more emphasis on the dissolved substances. On the other hand, this very modification may endow pinocytosis with still more interesting aspects, since it is brought into relation with the whole great problem of the active uptake and transport of substances by cells. It may well be that many of the more puzzling aspects of this problem will become clearer when we know more about the induction and the initial phases of pinocytosis.

This, therefore, would seem to be the most pressing problem for future investigation, and it seems to me that the fresh-water amoebae, with their adaptability and responsiveness to changing environments, are especially well suited for such studies.

References

1. LEWIS, W. H. 1931. Bull. Johns Hopkins Hosp. **49**: 17-27.
2. FREDERIC, J. & M. CHÈVREMONT. 1952. Arch. biol. **63**: 109-131; 259-277.
3. GEY, G. O., P. SHAPRAS & E. BORYSKO. 1954. Ann. N. Y. Acad. Sci. **58**(7): 1089-1109.
4. POMERAT, C. M., C. G. LEFEBER & MCD. SMITH. 1954. Ann. N. Y. Acad. Sci. **58**(7): 1311-1321.
5. PAUL, J. 1957. Demonstration. Tissue Conf. Glasgow, Scotland.
6. MAST, S. O. & W. L. DOYLE. 1934. Protoplasma. **20**: 555-560.
7. CHAPMAN-ANDRESEN, C. & D. M. PRESCOTT. 1956. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **30**: 57-78.
8. HOLTER, H. & J. M. MARSHALL, JR. 1954. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **29**: 7-26.
9. CHAPMAN-ANDRESEN, C. 1957. Lecture. 9th Intern. Congr. Cell Biol., St. Andrews, Scotland.
10. PALADE, G. E. 1956. J. Biophys. Biochem. Cytol. **2** (4) part 2 (Suppl.): 85-98.
11. BENNETT, H. S. 1956. J. Biophys. Biochem. Cytol. **2** (4) part 2 (Suppl.): 99-103.
12. MARSHALL, J. M., JR., V. N. SCHUMAKER & P. W. BRANDT. Ann. N. Y. Acad. Sci. **78**(2): 515-523.
13. LEWIS, W. H. 1937. Am. J. Cancer. **29**: 666-679.
14. CHAPMAN-ANDRESEN, C. & H. HOLTER. 1955. Exptl. Cell Research, Suppl. **3**: 52-63.
15. LØVTRUP, S. & A. PIGOÑ. 1951. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **28**: 1-36.
16. ANDRESEN, N., C. CHAPMAN-ANDRESEN, H. HOLTER & C. V. ROBINSON. 1953. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **28**: 499-528.
17. CHAPMAN-ANDRESEN, C. 1953. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **28**: 529-540.

Part III. Cytochemistry and Enzymes

MICROSCOPIC ENZYME CHEMISTRY OF CARBOXYLIC ESTERASES IN AMOEBAE*

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Introduction

A considerable body of knowledge concerning enzymes in amoebae has developed along microchemical, microtechnical, and presumptive lines of investigation.

Acid phosphatase activity has been investigated microcolorimetrically, in *Amoeba proteus* (Brachet, 1954a) and in *Pelomyxa carolinensis* (Holter, 1954b; 1955). The enzyme was nucleus-dependent (Brachet, 1954a). The enzyme dissociated from the mitochondria during "homogenization and fractionation" (Holter, 1955).

Adenosine triphosphatase activity has been investigated microcolorimetrically in *A. proteus* (Brachet, 1954a, b). The enzyme was not nucleus-dependent. Amylase activity has been investigated microiodometrically, in *A. proteus* (Holter and Doyle, 1938a, b; Urbani, 1952b; Brachet, 1954a). *In vivo* stratification displaced activity into the mitochondria-rich zone. A portion of the activity had somewhat greater stability and a higher pH-optimum than the remainder (Holter and Doyle, 1938b). The enzyme was not nucleus-dependent (Urbani, 1952b; Brachet, 1952). "Catalase" activity has been estimated, microiodometrically in *A. proteus* (Holter and Doyle, 1938b).

Cytochrome oxidase activity has been inferred from cyanide sensitivity, for *A. proteus* (Clark, 1942) and for *P. carolinensis* (Pace and Belda, 1944; Pace and McCashland, 1951). However, in homogenates of strata resulting from *in vivo* centrifugation of *P. carolinensis* (Andresen *et al.*, 1951) and *Physarum polycephalum* (Andresen and Pollock, 1952; Holter and Pollock, 1952) Cartesian diver microrespirometry failed to detect increases in oxygen uptake with succinate-cytochrome *c* (*Pelomyxa* and *Physarum*) or with ascorbate-cytochrome *c* (*Physarum*)—methods dependent on cytochrome oxidase activity. Cytochrome could not be identified spectroscopically in *Physarum* (Allen and Price, 1950) but cytochrome *a*, cytochrome *b*, and cytochrome *e* have been detected microspectroscopically in *A. proteus* and *P. carolinensis* (Moller and Prescott, 1955). More recently, both cytochrome oxidase and ascorbic acid oxidase have been detected in *Physarum* homogenates (Ward, 1958).

* This work is taken, in part, from a thesis presented to the faculty of the Graduate School of Arts and Science, New York University, in partial fulfillment of the requirements for a Ph.D. degree in the Department of Biology.

Dehydrogenase activity has been inferred from ethyl urethan sensitivity for *P. carolinensis* (Pace and Belda, 1944). Cartesian diver respirometry on *P. polycephalum* (Holter and Pollock, 1952; Andresen and Pollock, 1952), has revealed that one fourth of the oxygen utilization is due to dehydrogenase activity other than that of succinic dehydrogenase. Succinic dehydrogenase has been investigated in *P. carolinensis* by the same method (Andresen *et al.*, 1951; Holter, 1954a, b; Holter and Lumsden, 1954). *In vivo* stratification displaced activity into mitochondria-rich zones in all cases. Comparison between the usual "homogenization and fractionation" technique and homogenization of zones obtained by *in vivo* stratification revealed that succinic dehydrogenase activity was not separated from the mitochondrial fraction by "homogenization and fractionation" (Holter, 1954a; Holter and Lumsden, 1954).

Digestive activity has been investigated in *A. proteus* (Clark, 1943) by microscopic observation of food vacuoles and traumatized nuclei.

Dipeptidase activity has been investigated microchemically, in *A. proteus* (Holter and Kopac, 1937; Holter and Doyle, 1938b; Andresen and Holter, 1949; Urbani, 1952a; Brachet, 1954b), in *P. carolinensis* (Andresen and Holter, 1949; Holter and Løvtrup, 1950; Holter, 1954b), in *Pelomyxa palustris* (Andresen and Holter, 1949), and in *P. polycephalum* (Andresen and Pollock, 1952; Holter and Pollock, 1952). Activity was not displaced centrifugally and was presumed to reside in the matrix or microsome fraction. Differences in pH optima and in conditions for contact between enzyme and substrate were observed. Approximately one-half of the activity was not nucleus-dependent (Urbani, 1952; Brachet, 1954a). Comparison between the usual "homogenization and fractionation" technique and homogenization following *in vivo* stratification showed that dipeptidase activity was not separated from the microsomal fraction by "homogenization and fractionation" (Holter, 1954b).

Enolase activity has been investigated microchemically in *A. proteus* (Brachet, 1954a, b). The enzyme was not nucleus-dependent.

Esterase activity has been investigated microchemically, in *A. proteus* (Brachet, 1954a). The enzyme was nucleus-dependent.

Glycolytic activity has been investigated microcolorimetrically in nucleate and anucleate *A. proteus* (Brachet, 1950; Linet and Brachet, 1951). The degree of nucleus-dependence was not clear.

Lipase activity has been inferred for *Amoeba dubia* (Dawson and Belkin, 1928), *A. Proteus* (Dawson and Belkin, 1929), and *P. carolinensis* (Wilber, 1942) from microscopic and cytochemical observations on the fate of injected oils. Visible fatty inclusions in the cytoplasm of *P. carolinensis* appeared not to constitute an available energy source, there being little evidence of diminution even in terminal stages of starvation (Andresen and Holter, 1945). Lipase activity has been inferred for *A. proteus* from observations on the fate of fat in ingested *Chilomonas* (Mast and Hahnert, 1935) and *Colpidium* (Mast, 1938), and for *P. carolinensis* from the fate of fat in ingested *Colpidium* (Wilber, 1942). Observations on sudanophilia in nucleate and anucleate fragments of *A. proteus* suggested lack of nucleus dependency (Brachet, 1950).

Peroxidase activity has been investigated cytochemically in *A. proteus*

(Brachet, 1950). Comparison between nucleate and anucleate fragments suggested lack of nucleus dependency.

Phosphorylating activity has been inferred for *A. proteus* (Mazia and Hirshfield, 1950; Brachet, 1952) from the uptake of phosphorus-32. A most dramatic drop in uptake by anucleate fragments, not accompanied by a drop in oxygen uptake, was attributed to failure in the mechanism of coupling between oxidation and phosphorylation.

Proteinase activity has been investigated microphotometrically in *A. proteus* (Andresen and Holter, 1949; Urbani, 1952a; Brachet, 1954a), in *P. carolinensis* (Andresen and Holter, 1949; Holter and Løvtrup, 1949; Løvtrup, 1950; Holter, 1954a, b), and in *P. palustris* (Andresen and Holter, 1949). *In vivo* stratification displaced activity into mitochondria-rich zones. The activity became separated from the mitochondrial fraction during "homogenization and fractionation" (Holter, 1954a, b). Activity was not nucleus-dependent (Urbani, 1952a; Brachet, 1954a).

Protein-synthesizing activity and active transport have been inferred for *A. proteus* (Mazia and Prescott, 1955) from the uptake and incorporation of methionine sulfur-35. Methionine uptake suffered immediately in anucleate fragments in consequence of what was considered a nucleus-dependent failure of active transport, the percentage incorporation into protein remaining unchanged in the fragments.

Finally, carbohydrases, lipases, and proteinases serially activated during starvation have been inferred for *P. carolinensis* from Cartesian diver balance studies on the densities of substrates utilized with time (Holter and Zeuthen, 1948; Zeuthen, 1948a, b; Holter, 1949, 1950). Submicroscopic lipid (neutral fat and phospholipid) utilization was inferred for the middle period. The share of lipids in starvation metabolism was found to be surprisingly high. A few respiratory quotients were obtained during the middle period in the Cartesian diver microrespirometer; the quotient of between 0.75 and 0.88 was consistent with the view that lipids were important in the middle period. Of great interest was the increased coalescency between vacuoles following the middle period of starvation, in view of the role assigned to phospholipids in the maintenance of such protoplasmic membranes (Bungenberg de Jong and Bonner, 1935).

The present report deals with the preparation of these highly plastic cells for microscopic enzyme cytochemistry, the detection of several carboxylic esterases—esterases, lipases, and cholinesterase—and their validation, classification, and identification with the grosser protoplasmic subdivisions in *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis* under common conditions of laboratory culture. Concomitantly, debris and other organisms in these cultures were studied to ascertain the magnitudes of their contributions to the reactions in the amoebae, if any. The substrate esters utilized were primarily those entering into a principle of microscopic enzyme histochemical classification of carboxylic esterases (Gomori, 1952b). General references dealing with rationales and procedural details for the microscopic detection of enzyme activities are the textbooks of Gomori, 1952a; Lison, 1953; and Pearse, 1953.

Materials and Methods

Experimental animals. *A. dubia* and *A. proteus* were obtained from the laboratories of J. A. Dawson of The College of the City of New York, New York, N. Y., and M. J. Kopac of New York University, New York, N. Y.; *P. carolinensis* was obtained from the laboratory of Dawson and from subcultures maintained in the laboratory of Kopac. *P. illinoisensis* was obtained from the laboratory of E. W. Daniels of the Argonne National Laboratory, Chicago, Ill., and from subcultures maintained in the laboratory of Kopac.

Culture. The inorganic milieu was that of Brandwein, 1935. Agar was omitted (Heller, 1952). Cultures were maintained in a water-cooled incubator at temperatures varying between 18 and 23° C., and subcultures were made every 30 days. Food organisms were variably *Blepharisma*, *Chilomonas*, *Colpidium*, small and large euglenoid forms (resembling *Peranema* and *Euglena*), small and large cirrous ciliates (resembling *Euplotes*, *Oxytricha* and sometimes *Stylonychia*), *Paramecium*, and *Spirostomum*. Also ingested were several types of rotifers (resembling *Philodina*, *Monostyla*, and *Anuraca*), at least two kinds of mold spores (the presence of *Dictyuchus* and *Saprolegnia* is reported by Dawson, 1955, in his *Amoeba* cultures) and, occasionally, a whole ascus, a plant filament, or a microworm. The annelid *Aeolosoma* was present in variable numbers feeding on bottom debris consisting of particulates, monads (round forms with single flagella and reddish-brown granules), and bacteria. On one occasion sperm, presumably arthropod, nematode, or rotifer sperm, were found embedded in bottom debris. Several types of tiny amoeboid forms were also present in variable numbers (*Amoeba doffeini* is reported by Dawson, 1955, in his *Amoeba* cultures). Ova were observed in some *A. proteus* cultures.

Selection. Selection was made, as much as possible considering the large numbers processed, for similarities in degree of attachment, location in culture, shape, size, and transparency.

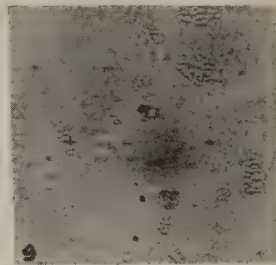
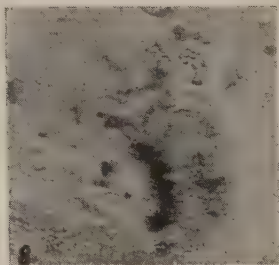
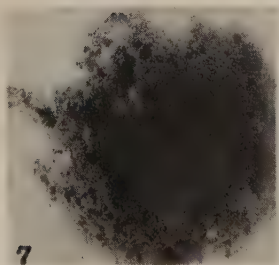
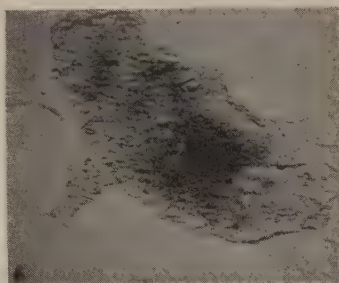
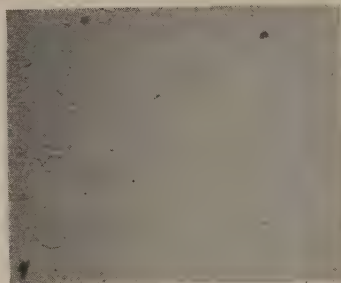
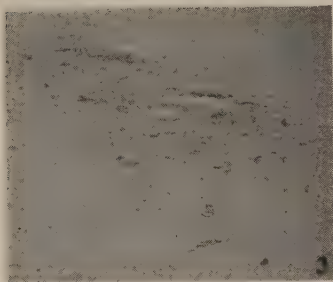
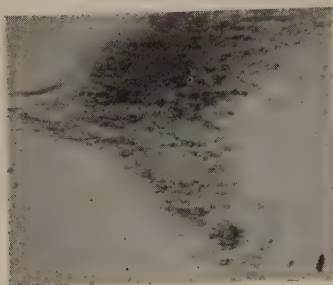
Washing. This was accomplished by serial passage through Brandwein's solution.

Flattening. Active flattening on a coverslip in a moist storage chamber was generally complete in 30 to 60 min.

Primary fixation. Cover slips bearing the washed and flattened amoebae were plunged into acetone at approximately 4° C. and stored at the same temperature overnight. Food organisms and debris were fixed in the same way. The primary fixation was instantaneous compared to fixation in 10 per cent formalin.

With beta-naphthyl acetate and the acetate of 2-hydroxy 3-naphthoic acid anilide as substrates for esterase activity, preferred methods of fixation involving acetone are given as cold acetone (Lillie, 1954), cold acetone followed by double embedding (Pearse, 1953), and cold acetone followed by paraffin (Gomori, 1952a).

With the monostearate (Tween 60) and with the monooleate (Tween 80) esters of polyoxyethylene sorbitan as substrates for lipase activity, preferred methods of fixation involving acetone are given as cold acetone (Lillie, 1954) and cold acetone followed by double embedding (Gomori, 1952a).



With myristoyl choline as substrate for cholinesterase activity, preferred methods of fixation involving acetone are given as cold acetone (Lillie, 1954), cold acetone followed by double embedding (Pearse, 1953), cold acetone followed by double embedding as for lipase (Gomori, 1948), and cold acetone as for esterase (Gomori, 1952a).

The beta-naphthyl acetate-diazonium salt system was used to test several other types of fixation. Fixation in acetone alone resulted in rather weak reactions (FIGURE 1) due probably to esterase diffusibility (Doyle and Liebelt, 1954). With briefer fixation the deposition tended to be coarser. Reaction intensities were markedly improved by secondary fixation in formalin (FIGURE 2). Negative or feeble reactions resulted when the animals were dried on cover slips without fixation (Auerbach, 1953) and after alcohol fixation (FIGURE 3) or 5 per cent acetic acid in alcohol (FIGURE 4); fixation in alcohol containing formalin resulted in intense reactions (FIGURE 5), but these were patchy and gradient, due possibly to some balance between enzyme inactivation (Pearse, 1953) or elution (Folch and Lees, 1950; Nachlas *et al.*, 1957) by the alcohol and immobilization by the formalin.

Formalin in acetone produced weak, patchy intensities (FIGURE 6) which could be attributed to a sparingly soluble polymer formed in such a mixture and left behind in the drying animals.

Affixing. After overnight fixation in cold acetone, the animals in the acetone appeared somewhat opaque, granular, and grayish-brown. Larger inclusions were readily seen under the dissecting microscope, and all outlines appeared clear and sharp. Variable numbers of amoebae lay free on the bottom of the dish, others remaining lightly attached to the cover slip to which they had attached themselves in life. Most of the animals remaining attached were easily loosened without fracture by means of an undercurrent of acetone directed against them with the hand micropipette. They were then picked up with the hand micropipette and transferred to clean cover slips. In a few seconds the animals were dry and appeared as specks of white ash to the eye. In this state they transmitted very little light and appeared quite black under the dissecting microscope. When processed in this dehydrated state, severe losses were experienced, especially with the Gomori procedure in which demineralization, more prolonged incubation, more steps—especially the sulphiding step—and more washing, all contributed to decreased recovery. After rehydration with a drop of 90 to 95 per cent acetone followed by secondary drying, the animals appeared waxy to the eye and were quite transparent under the dissecting

←

FIGURES 1 to 6. Reactions of *A. dubia* with beta-naphthyl acetate. $\times 290$. (1) Weak reactions after acetone fixation. (2) Strong reactions after primary fixation in acetone, followed by secondary fixation in formalin. (3) Negligible reactions after fixation in alcohol. (4) Negligible reactions after fixation in alcohol containing 5 per cent acetic acid. (5) Strong but patchy gradient and leached reactions after fixation in alcohol containing formalin. (6) Weak patchy reactions after fixation in acetone containing formalin. FIGURES 7 to 9. Endogenous cation in *A. dubia* capable of exchange with lead, incubation by-passed. $\times 290$. (7) Without demineralization, cation is present in refractile bodies and cytoplasm. (8 and 9) Efficient demineralization after 15 min. in citrate buffer and Versene, respectively, followed by 1 min. in water; refractile bodies disappear, but chilomonad bodies remain.

microscope. Food organisms and debris were handled in the same manner and on the same cover slip as that of the amoebae from whose cultures they were taken.

Secondary fixation. Fixation with acetone alone did not result in reaction intensities as high as when cold formalin was included in the process. Formalin solutions were prepared and chilled at approximately 4° C. for approximately 1 hour before introduction of cover slips with affixed amoebae. This procedure was usually observed because of unpredictable polymerization changes said to follow dilution of formaldehyde solutions (Walker, 1953) and because of as-yet unpredictable interreactions between formaldehyde and proteins (French and Edsall, 1945).

Formalin Concentrations

For experiments involving beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide.....	10 per cent
For experiments involving the monostearate and monooleate esters of polyoxyethylene sorbitan.....	10 per cent
For experiments involving myristoyl choline.....	6 per cent

With beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide as substrates for esterase activity, preferred methods of fixation involving formalin are given as cold formalin (Gomori, 1952a; Lillie, 1954), cold formalin followed by frozen section (Seligman *et al.*, 1951), and cold formalin followed by gelatin embedding and frozen section (Pearse, 1953).

The cover slips were immersed in the formalin in Columbia dishes that were covered and returned to the refrigerator for storage overnight. Fixation for shorter periods resulted in little advantage. Fixation for less than two hours resulted in coarser deposition. Whether the enzyme had been activated by the formalin or whether some favorable balance between inactivation and resistance to extraction had resulted would be hard to say in our present state of knowledge. The carboxylic esterases are dependent on the integrity of some of their sulfhydryl groups (Ammon and Jaarma, 1950; Barron, 1951; Zacks, 1955), and such groups may be both liberated and bound by formaldehyde treatment, depending on time, temperature, and concentration, in an as yet unpredictable manner (French and Edsall, 1945). Polysulfide in the primary fixative intensified the reactions, but in the secondary fixative greatly weakened them. Cysteine weakened the reaction, as did iodoacetate. There was little difference between 5 per cent and 10 per cent formalin, except with the myristoyl choline substrate with which the higher concentration resulted in decreased eserine sensitivity.

Postformalin washing. The amoebae were washed 15 to 30 min. in running water.

General-inactivation controls. Cover slips containing animals to be so inactivated were placed in Columbia dishes submerged in boiling water for fifteen minutes, in Columbia dishes containing 5 per cent phenol for 15 min., or in Columbia dishes containing Lugol's iodine for 15 min. All other cover slips remained in Columbia dishes in running water.

Postgeneral-inactivation washing. Columbia dishes containing the general inactivation controls were rinsed and placed in running water "downstream" from the other Columbia dishes for 30 min.

Demineralization. This is essential in the Gomori type of procedure where

endogenous insoluble cation would be confused with cation precipitated at sites of enzyme activity (FIGURE 7). Demineralization was accomplished in a 0.2-M citrate buffer (pH 4.5 for 15 min.), which now replaced the water in all Columbia dishes (FIGURE 8). Some experiments pointed to the efficacy (and enzyme compatibility) of 0.001 M disodium ethylene diamine tetra-acetic acid dihydrate in water (FIGURE 9).

Postdemineralization washing. All Columbia dishes were rinsed and placed in running water for 15 min. in experiments involving the monostearate and monooleate esters of polyoxyethylene sorbitan and the myristate ester of choline.

Preincubation inhibition (or activation). Columbia dishes containing animals to be exposed to chemicals before incubation only and Columbia dishes containing animals to be exposed to chemicals before incubation, as well as during incubation, were emptied of water and filled with appropriate solutions. The higher concentrations of sodium taurocholate and of quinine hydrochloride were somewhat higher in pH than was the solvent, distilled water, but in no case was neutrality exceeded. All other Columbia dishes were filled with distilled water at room temperature. In general, two concentrations of each chemical were used, these concentrations being similar to those used by Gomori and Chessick; the chemicals used were those considered useful for their selective effects on carboxylic esterases (Gomori, 1948; Gomori, 1952b; Gomori and Chessick, 1953). Gomori and Chessick used these chemicals during incubation only. In the present report they were generally used before incubation only, during incubation only, and both before and during incubation. Preincubation exposure times were 30 min. in experiments involving beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide and 60 min. in experiments involving myristoyl choline and the monostearate and monooleate esters of polyoxyethylene sorbitan.

The concentrations of arsenilic acid used by the above workers were 2×10^{-4} M and 10^{-3} M. These concentrations were used in the present report.

The concentrations of eserine and of prostigmine used by the above workers were 10^{-5} M to 10^{-4} M. Concentrations of this class of inhibitors used in the present report were variably 10^{-6} M, 2×10^{-6} M, 10^{-5} M, and 10^{-4} M.

The concentrations of fluoride used by the above workers were 2.5×10^{-4} M to 2×10^{-3} M. These concentrations were used in the present report.

The concentrations of quinine used by the above workers were 5×10^{-3} M to 10^{-1} M. The concentrations used in the present report were 5×10^{-3} M and 2×10^{-2} M.

The concentrations of taurocholate used by the above workers were 2×10^{-3} M to 2×10^{-2} M (Gomori and Chessick, 1953). The concentrations used in the present report were 2×10^{-3} M and 2×10^{-2} M.

Postpreincubation inhibition (or activation) washing. The Columbia dishes were rinsed and placed in running water downstream from the Columbia dishes which had not contained chemicals for 60 min. in experiments involving myristoyl choline and the monostearate and monooleate esters of polyoxyethylene sorbitan and for 15 min. in experiments involving beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide.

Incubation. Suitable incubation times were determined in preliminary experiments. Incubation was carried out for 20 min., with constant agitation at the temperature of an ice-water mixture, in experiments involving beta-naphthyl acetate; for 60 min. at room temperature in experiments involving the acetate of 2-hydroxy-3-naphthoic acid anilide; for 36 hours at 37° C. in experiments involving myristoyl choline; and for 48 hours at 37° C. in experiments involving the monostearate and monooleate esters of polyoxyethylene sorbitan.

Columbia dishes containing animals that were not to be exposed to inhibitors (or activators) and Columbia dishes containing animals that were to be exposed to inhibitors (or activators) only before incubation were now filled with normal media. Columbia dishes containing animals that had not been exposed to these chemicals, but that were to be exposed to them only during incubation, and Columbia dishes containing animals that had been exposed to these chemicals before incubation and that were to be exposed to them during incubation as well were now filled with normal media containing the different concentrations of the different chemicals.

Additional controls consisted of animals incubated in media deficient in one or more ingredients essential to the formation of the insoluble reaction product.

Due to the low solubility products of the fluorides of calcium and of cobalt, fluoride was not included during incubation in media for the demonstration of activity against myristoyl choline and the monostearate and monooleate esters. In these Gomori-type media, calcium or cobalt ions are essential ingredients of the precipitation process during normal incubation. In some cases, however, it was found that the fatty acids liberated (myristic acid and stearic acid) were sufficiently insoluble and of suitable melting point, relative to the temperature of incubation, to precipitate somewhat without cation; in such cases fluoride was incorporated into a cation-free medium for comparison.

Nonspecific coupling between diazonium salts and reactive protein groups such as the tyrosine phenolic, the tryptaphan indole, and the histidine iminazole, usually results in pale yellow coloration, although with some diazonium salts more intense coloration is possible (Pearse, 1954). Under normal conditions of incubation, nonspecific coloration with alpha-naphthyl diazonium naphthalene 1,5-disulfonate was found to be of the paler variety (both specific and nonspecific coloration with Diazo Blue B, Diazo Red RC, and anthraquinone diazonium chloride were less satisfactory). Nevertheless, it occurs to one that if the diazonium salt were to attach itself to a site unrelated to enzyme activity, and in such a manner as to leave reactive the diazonium group that couples with the product of enzyme activity, the possibility for false localization exists. It was found, however (FIGURES 10 to 12), that: (1) despite the intensity of the specific reactions in the food vacuoles and in the cytoplasm following normal incubation, the food vacuoles and the cytoplasm were completely negative for specific coloration in the absence of either substrate or diazonium salt; (2) the amount of naphthol retained by these large cells, after 20 min. of substrate hydrolysis in the absence of diazonium salt, was insufficient for detectable specific coloration when followed immediately by diazonium coupling; (3) the amount of free diazonium salt remaining in these large cells after immersion for 20 min. in medium containing diazonium salt, but lacking substrate,

was insufficient for detectable specific coloration when followed immediately by liberation of naphthol from the substrate; (4) although nonspecific coloration and therefore nonspecific diazonium salt binding were in evidence following incubation in a medium containing diazonium salt, but lacking substrate, there was no evidence of specific coloration on subsequent incubation in a medium containing substrate but lacking diazonium salt, which showed that there was no danger of false localization on the score of nonspecific binding of diazonium salt; (5) the results suggested also that a very great turnover coupled with simultaneous precipitation was necessary before the accumulation threshold of microscopic visibility of the specific coloration could be crossed.

Postincubation washing. Columbia dishes were rinsed and placed in running water for 15 min. in experiments involving beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide; for 30 min. in experiments involving the monostearate and monooleate esters of polyoxyethylene sorbitan; and for 60 min. in experiments involving myristoyl choline.

Postincubation washing times vary in the literature. The above washing times were chosen after tests for maximum reduction of background darkening of the inactivated controls. With the Gomori types of media, failure to remove unbound cation normal to the incubation process was found to be one of the causes of nonspecific coloration. Cobalt was found more difficult to remove than calcium, accounting for the longer postincubation washing time with myristoyl choline.

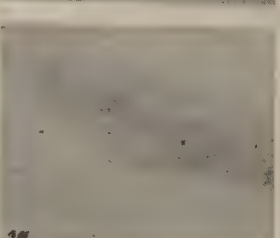
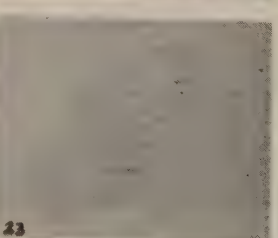
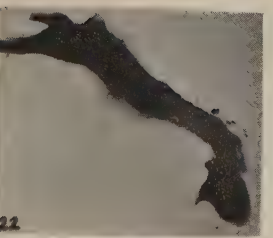
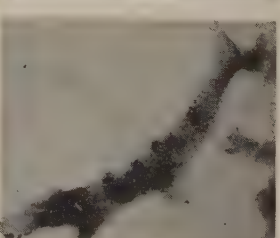
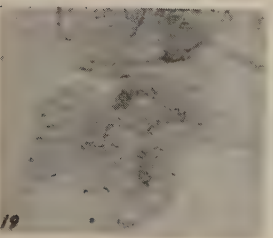
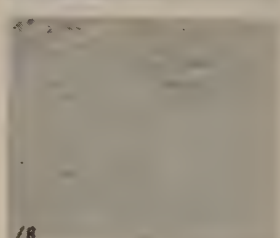
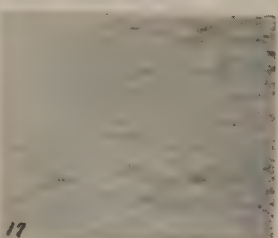
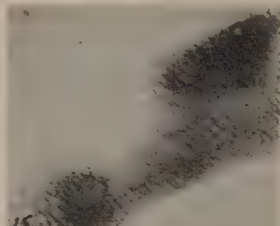
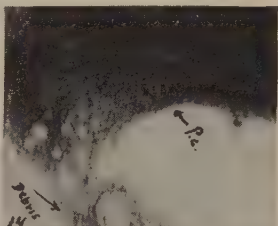
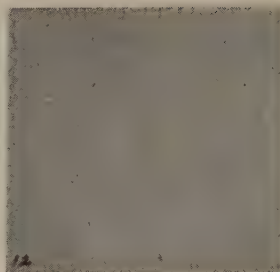
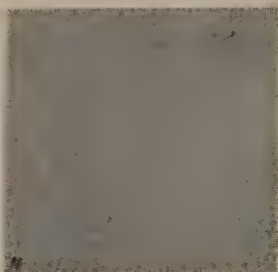
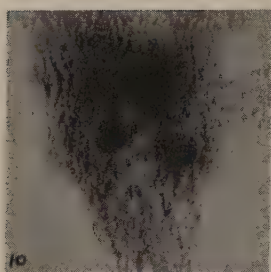
Cation exchange. Columbia dishes were filled with 2 per cent lead nitrate in experiments involving the monostearate and monooleate esters of polyoxyethylene sorbitan.

One per cent lead nitrate for 15 min. at room temperature is given by Pearse (1953), and 1 to 2 per cent lead nitrate for 10 min. at the temperature of the paraffin oven is given by Gomori (1952a). The higher concentration was borrowed from Gomori, and the lower temperature and more prolonged exchange time were borrowed from Pearse, to avoid fracture and excessive losses of animals from cover slips, which occurred with abrupt temperature changes.

Cation exchange is not necessary in experiments involving myristoyl choline since the insoluble cobalt soap is visualized as a sulfide of cobalt. There is no cation exchange involved in experiments with beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide since an insoluble azo dye is laid down during incubation.

Postcation-exchange washing. Where cation exchange was necessary, as in the experiments involving the monostearate and monooleate esters of polyoxyethylene sorbitan, Columbia dishes were then placed in running water for 30 min. Running water for 5 min. is cited by Pearse (1953) and repeated changes of distilled water by Gomori (1952a). In the present investigation, 30 min. in running water was found necessary for maximum reduction of background darkening of the inactivated controls.

Anion exchange. Columbia dishes were filled with a solution of 5 drops of light yellow ammonium sulfide in 100 ml. of water. Stronger solutions seriously impaired recovery of animals from the ensuing rinsing and washing. Immersion was for 10 min. at room temperature in experiments involving the



monostearate and monooleate esters of polyoxyethylene sorbitan and for 15 min. at room temperature in experiments involving myristoyl choline.

One to 2 min. at room temperature is cited by Pearse (1953) and 5 min. at room temperature by Gomori (1952*a*) for the first 2 esters; 1 to 2 min. at room temperature is cited by Pearse (1953) and 15 min. at room temperature by Gomori (1948), for the third ester.

Postanion-exchange washing. Columbia dishes were rinsed as gently as possible, since considerable losses tended to occur at this time, and were left in running water for at least 5 min. before mounting. In experiments involving beta-naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide, this procedure was followed directly after incubation, the intervening steps being unnecessary.

Mounting. All mounting was in Kaiser's glycerine jelly. Curiously enough, clearing in xylol in preparation for balsam mounting faded the metallic sulfides; the azo dyes formed from beta-naphthol and from 2-hydroxy-3-naphthoic acid anilide are soluble in lipid solvents. Euparal and diaphane after 95 per cent alcohol faded the metallic sulfides in a few hours. Glychrogel resulted in greater contrast and was compatible with the metallic sulfides for a suitable period of time, but deterioration of the azo dyes was noted after several days. The glycerin jelly was compatible with the metallic sulfides and with the azo dyes for a suitable period of time.

Microscopic examination. Incubation with beta-naphthyl acetate and with the acetate of 2-hydroxy-3-naphthoic acid anilide in the presence of alpha-naphthyl diazonium naphthalene 1,5-disulfonate (Manheimer and Seligman, 1948) resulted in specific shades of reddish purple and bluish purple, respectively, against nonspecific light ochre shades. With the monostearate and monooleate esters of polyoxyethylene sorbitan the specific coloration was a warm brownish-black. With myristoyl choline, colder shades of brownish black resulted.

Rating of reaction intensities. Reactions were rated at 580X through the horizontal reflex ocular of the Leitz Micro-Ibso photomicrographic attachment. Color comparator wheels with 10 graded segments were mounted 12 inches in front of the microscope so as to be superimposed on the microscopic field of

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FIGURES 10 to 12. Reactions of *A. dubia* in beta-naphthyl acetate deficiency media, followed by complementary media; positive reactions with beta-naphthyl acetate only when diazonium salt is simultaneously present. X290. (10) Strong positive reaction in normal medium. (11) Negative reaction in medium lacking diazonium salt followed by medium lacking beta-naphthyl acetate. (12) Negative reaction in medium lacking beta-naphthyl acetate, followed by medium lacking diazonium salt. FIGURES 13 to 16. Normal reactions in *P. illinoisensis*, *P. carolinensis*, *A. proteus*, and *A. dubia*, respectively, with beta-naphthyl acetate; deposition noted in food vacuoles and cytoplasm, but not in nuclei. X290. FIGURES 17 to 19. General inactivation procedures prior to incubation with beta-naphthyl acetate; boiling water (*P. illinoisensis*), phenol (*A. proteus*), and iodine (*A. dubia* with some residual activity), respectively. X290. FIGURE 20. Arsenilate inhibition of *P. illinoisensis* with beta-naphthyl acetate. X290. FIGURE 21. Taurocholate inhibition of the cytoplasm with little effect on the food vacuoles in *P. illinoisensis* with beta-naphthyl acetate. X70. FIGURE 22. Quinine intensification in *P. illinoisensis* with beta-naphthyl acetate. X70. FIGURE 23. Negative reactions in large cirrous ciliate and chilomonad with normal beta-naphthyl acetate incubation. X290. FIGURE 24. Small cirrous ciliate and *Paramecium*; positive reaction in *Paramecium* with normal beta-naphthyl acetate incubation. X290.

vision when viewed with the eye not engaged at the ocular. The grades of intensity on the color wheel tended to be logarithmic. The color wheels provided a reasonable degree of standardization, at least at a perceptual level, leading to a reasonable degree of reproducibility in evaluating intensities as a result of enzyme activity.

The "complete" experiment. A "complete" experiment with a given substrate was envisioned as including a simultaneous run with *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis*, together with other organisms and debris from each culture.

In the same complete experiment were to be included the effects of arsenilate, eserine, fluoride, quinine, and taurocholate, each at two levels of concentration. The chemicals and concentrations were those recommended in the literature as of value in distinguishing between the classes of enzymes. The general consensus at this time is that the degree of diagnostic success at the level of biochemical enzymology has not carried over to the level of microscopic histochemical enzymology. For this reason the use of these chemicals in the present investigation was extended to include treatment with each chemical at each concentration under conditions of progressive severity—treatment prior to incubation only, treatment during incubation only, and treatment prior to as well as during incubation. With beta-naphthyl acetate, only the third type of treatment proved feasible because of the rapidity of esterolysis. In the same complete experiment were to be included such controls for purposes of validation and comparison as seemed to be required by the nature of the microscopic enzyme cytochemical process involving a given substrate.

The 5 esters used in the 5 complete experiments were to be those entering into a principle of classification of carboxylic esterases (Gomori, 1952*b*) beta naphthyl acetate and the acetate of 2-hydroxy-3-naphthoic acid anilide for 2 simple esterases, the monostearate and monooleate esters of polyoxyethylene sorbitan for 2 lipases, and myristoyl choline for cholinesterase. Some subsidiary experiments involved related esters.

Results

Beta-Naphthyl acetate. Microscopic enzyme chemistry, as it applies to this ester, depends on precipitation of an insoluble azo dye as the result of combination between diazonium salt present in the medium and enzymatically liberated beta-naphthol.

The species, in decreasing order of both food vacuole and cytoplasm intensities were *P. illinoisensis*, *P. carolinensis*, *A. proteus*, and *A. dubia* (FIGURES 13 to 16). Specific deposition was not observed in the nuclei. Pretreatment with boiling water abolished all specific coloration (FIGURE 17). Pretreatment with 5 per cent phenol or with Lugol's iodine abolished specific coloration (FIGURE 18) or rendered specific coloration barely perceptible (FIGURE 19). The percentages of change from normal of the numerical averages of the food vacuole and cytoplasm intensities in the 4 species of amoebae, following treatment with chemicals both prior to and during incubation, are shown in TABLE 1.

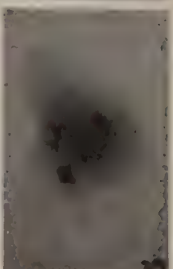
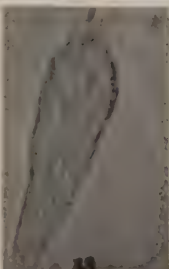
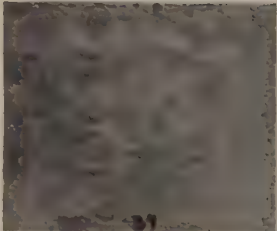
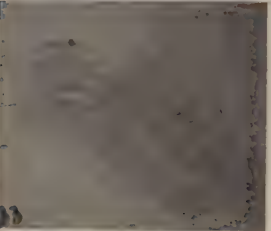
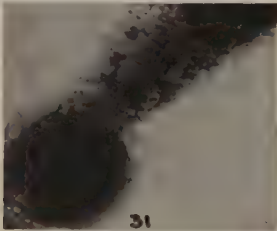
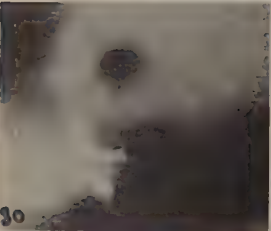
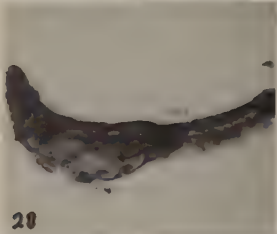
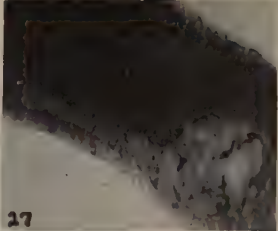
The most noteworthy effects appeared to be the very considerable inhibition

TABLE 1

	Food vacuoles				Cytoplasm			
	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>
Arsenilate								
2×10^{-4} M	-47	-28	-23	-28	-45	-26	-21	-37
10^{-3} M	-62	-48	-44	-44	-60	-52	-41	-42
Eserine								
10^{-6} M	+5	+9	-1	-10	+2	-8	-1	-8
10^{-4} M	-17	-16	-2	-17	-15	-18	-1	-25
Fluoride								
2.5×10^{-4} M	+3	+5	-5	-1	-2	0	-12	-6
2×10^{-3} M	+7	0	-1	-11	+7	-2	-4	-12
Quinine								
5×10^{-3} M	-2	+15	-7	+3	+7	+28	-9	+4
2×10^{-2} M	0	+4	-7	-13	+5	+12	-7	-19
Taurocholate								
2×10^{-3} M	+20	0	-2	-4	+18	-4	-4	-8
2×10^{-2} M	-18	-15	-8	+1	-55	-26	-14	-23

by arsenilate (FIGURE 20), the generally considerable inhibition by taurocholate (especially of the cytoplasm [FIGURE 21]) and a tendency toward activation (especially of the cytoplasm [FIGURE 22]) by quinine. Eserine, in a concentration effective against cholinesterase, and fluoride produced effects probably not to be regarded as significant.

Chilomonads were almost always negative (FIGURE 23). Cirrous ciliates, large (FIGURE 23) and small (FIGURE 24), were almost always negative, but cirrous ciliates observed in food vacuoles were frequently intensely stained. Colpidia and small and large euglenoids were negative to minimal when normally processed; with quinine, intensification was occasionally noted. Paramecia were moderately stained when normally processed (FIGURE 24), but were more intensely stained within amoeba food vacuoles (FIGURE 25). Rotifers were negative to minimal, but were more intense when observed within *Pelomyxa* (FIGURE 26). Small amoeboid forms and microworms were occasionally observed on cover slips after processing; amoebae have been reported in the "plasma" of *A. proteus* (Wolska, 1949) and therefore cannot be entirely eliminated in considering contributions by other organisms to the reactions in the amoebae. The annelid *Aeolosoma*, feeding on bottom debris, was intensely colored throughout its length after normal processing, although the intensities were somewhat diminished in the more transparent middle third, housing an expansion of the gut (FIGURE 28); specific coloration was markedly intensified by quinine (FIGURE 29). After iodine pretreatment, *Aeolosoma* was completely negative despite the fact that in *P. illinoisensis* from the same culture, as well as in *P. carolinensis*, considerable intensities remain in both food vacuoles and cytoplasm, this being of added significance, since monads and bacilluslike bodies were observed in the gut of *Aeolosoma* from the same culture. A similarly disproportionate effect was observed with the higher concentration of fluoride; the intensities in *Aeolosoma*, unlike those in the amoebae in which 2×10^{-3} M



fluoride produced little effect, were considerably reduced throughout. Debris, consisting of granular and amorphous material in which were embedded bacteria, monads and, occasionally, larger poorly defined organisms, was never negative after normal processing, although the intensities were generally minimal to little more than minimal; specific coloration was abolished by pre-treatment with heat, occasionally minimal after iodine and after phenol; reduced by arsenilate and eserine, especially at the higher concentrations; greatly reduced by fluoride concentrations that had little effect on the amoebae; occasionally increased with the lower concentration and generally decreased with the higher concentration of quinine; and generally reduced with taurocholate, especially with the higher concentration. In general, it appeared that the reactions observed in the food vacuoles and in the cytoplasm of *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis* could not be satisfactorily accounted for by bacteria, debris, or food organisms present in their cultures.

While the intensities were in part dependent on thickness, which may account in part for the greater intensities in the pelomyxae as compared with the amoebae, the greater intensities in *P. illinoisensis* as compared with *P. carolinensis* would not be explained on this basis.

A comparison between intensities of several organisms and of debris, simultaneously processed and expressed as grades on the color comparator wheel, and the thicknesses of these, expressed as units engraved on the fine adjustment of the microscope, reveals that there is no necessary relation between thickness and intensity of specific coloration* (TABLE 2).

Considerable evidence for sharp localization and against diffusion is seen in the sharpness with which recognized morphologic outlines, such as those of food organisms, are rendered in terms of specific coloration, outlines that may

* Absolute thicknesses, from measurements made by focusing on the upper and lower surfaces, with the aid of the calibration on the fine adjustment knob, are subject to complications from the various refractive indices in the light path, the shapes of entrance and exit surfaces, tube length and other complications that suggest that this method should not be used unless it cannot be avoided or unless rough figures are required (Galbraith, 1955).

FIGURE 25. *Paramecium* in *P. carolinensis* food vacuole, intensely stained with beta-naphthyl acetate. $\times 290$. FIGURE 26. Stained rotifer in *P. carolinensis*; staining of rotifers negative to minimal, with beta-naphthyl acetate, when taken from culture. $\times 290$. FIGURE 27. Large ingested organisms may demonstrate lack of activity although surrounded by intensely active cytoplasm (*P. illinoisensis*). $\times 290$. FIGURE 28. *Aeolosoma*, normal reactions with beta-naphthyl acetate. $\times 70$. FIGURE 29. *Aeolosoma*, quinine intensification with beta-naphthyl acetate. $\times 70$. FIGURES 30 to 33. Normal reactions in *P. carolinensis*, *P. illinoisensis*, *A. dubia*, and *A. proteus*, respectively, with naphthol AS acetate; deposition noted in food vacuoles and cytoplasm, but not in nuclei. The reactions in *A. proteus* are exceptionally weak. $\times 290$. FIGURE 34. Crushed *A. dubia*, showing chilomonads reacting with naphthol AS acetate under the influence of quinine. $\times 290$. FIGURE 35. Cirrus ciliate under cover-slip pressure in *P. illinoisensis* food vacuole; regional cytoplasm reaction with naphthol AS acetate is inhibited by taurocholate. Cirrus ciliates were almost always negative when taken from culture. $\times 290$. FIGURE 36. Anterior end of *Aeolosoma* showing low level activity with naphthol AS acetate in stomal and poststomal region. $\times 290$. FIGURES 37 and 38. Positive reaction in *Paramecium* and negative reaction in *Spirostomum* with naphthol AS acetate. $\times 290$. FIGURE 39. A very tiny amoeboid form staining intensely with naphthol AS acetate; the thinnest portion still showing coloration is about one tenth the thickness of *Spirostomum* which shows no coloration at all. $\times 290$.

TABLE 2

	Intensity		Thickness
	Cytoplasm	Food vacuoles	
<i>P. carolinensis</i>	8.0	7.0	5.5 (FIGURE 14)
<i>A. proteus</i>	7.0	8.0	3.0 (FIGURE 15)
Large cirrous ciliate from <i>A. proteus</i> culture	0.0		3.0 (FIGURE 23)
Granular debris from <i>P. carolinensis</i> culture		1.0	2.5 (FIGURE 14)
<i>Paramecium</i> from <i>P. carolinensis</i> culture	2.0		2.0 (FIGURE 24)

be those of lightly stained food organisms in darkly stained cytoplasm (FIGURE 27) or lightly stained and darkly stained food organisms in the same area of cytoplasm (FIGURE 13).

Beta-Naphthyl Laurate

Beta-naphthyl laurate has been used in the estimation of carboxylic esterase activity in tissue homogenates (Nachlas and Seligman, 1949); incubation for 1 hour at 37° C., followed by postincubation coupling with diazonium salt, despite the solubility of the primary reaction product, was possible because the enzyme determination was at a biochemical level, the product remaining in the "test tube." It seemed interesting to try to adapt it to microscopic enzyme cytochemical use with the amoebae.

Specific coloration was observed in neither food vacuoles nor cytoplasm after incubation at room temperature for as long as 2 hours, or after incubation at the temperature of the refrigerator for 1 to 24 hours. Nonspecific coloration became increasingly deeper and more troublesome. Postincubation treatment with 1 per cent hydrochloric acid in 80 per cent alcohol relieved some of the nonspecific coloration, but revealed no specific component.

In some animals, after 1 hour, there was, it is true, an impression of slight coldness of tone in the food vacuoles: an indication, perhaps, of slight specific activity. With the more prolonged incubations, the nonspecific coloration could have masked slight existing specific coloration; for this reason an entirely different approach with beta-naphthyl laurate was tried, that of competition with beta-naphthol acetate for esteratic sites on the enzyme molecule. However, there was little difference in the intensities produced with beta-naphthyl acetate alone and with beta-naphthyl acetate containing beta-naphthyl laurate in addition.

If affinity of beta-naphthyl acetate enzyme or enzymes for the laurate ester exists, it appeared to be of very low order, judging both from direct microscopic enzyme cytochemistry with beta-naphthyl laurate and the indirect use of beta-naphthyl laurate by an adaptation of the competing substrate technique. The two tests do not necessarily show the same thing. The tests might be considered mutually supporting if there were an overlap in substrate specificity with respect to the long and short chain esters. The facts of high

affinity for the acetate ester and low affinity for the laurate ester and, as will be seen, high affinities for the still longer chained stearate and oleate esters, seem to support the idea of a separation between the enzymes that split the acetate ester on the one hand and the stearate and oleate esters on the other. This information seemed to be of sufficient importance to continue with the competing substrate technique utilizing decreasing concentrations of the acetate. With $0.02 \times$ the normal concentration of beta-naphthyl acetate, specific deposition was barely visible but unmistakably present in the food vacuoles; with this concentration of beta-naphthyl acetate in the presence of the normal concentration of beta-naphthyl laurate, however, the presence of specific deposition was questionable. A low degree of affinity for the laurate ester may therefore have been indicated.

The acetate of 2-hydroxy-3-naphthoic acid anilide (naphthol as acetate). Microscopic enzyme chemistry as it applies to this ester depends on the precipitation of an insoluble azo dye as the result of combination between diazonium salt present in the medium and enzymatically liberated 2-hydroxy-3-naphthoic acid anilide.

The species, in decreasing order of food vacuole and cytoplasm intensities, were *P. carolinensis*, *P. illinoisensis*, *A. dubia*, *A. proteus*, the reactions in *A. proteus* being exceedingly faint. The cytoplasmic intensities in *A. dubia* were frequently similar to those in the pelomyxae. Specific deposition was not observed in the nuclei. The reactions were intensified in the presence of 5×10^{-3} M quinine during incubation except for a slight diminution in the food vacuoles of *P. carolinensis* (FIGURES 30 to 33). With beta-naphthyl acetate, the reactions had appeared quite intense in *A. proteus*; more intense, in fact, than in *A. dubia*. This inverse relationship with respect to the two esters is highly significant for the duality (Gomori, 1952a, b) of the enzymes splitting beta-naphthyl acetate and naphthol AS acetate, a duality admitted cautiously by some, for "the difference in most tissues is not so great as to warrant this supposition" (Pearse, 1953). Complete abolition of specific coloration was obtained in all species with some form of arsenilate treatment. The percentages of change from normal of the numerical averages of the food vacuole and cytoplasm intensities in the four species of amoebae, following treatment with chemicals both prior to and during incubation are shown in TABLE 3.

Treatment with chemicals during incubation only produced very much the same results except that quinine was more generally activating:

Quinine	Food vacuoles				Cytoplasm			
	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>
5×10^{-3} M	+37	+50	-10	+40	+12	+114	+25	+42
2×10^{-2} M	-10	+63	-1	+12	-25	+86	+15	+38

Fluoride, however, inhibited without exception. The effects of treatment prior to incubation only were less marked, but arsenilate inhibition was still notable.

Blepharisma was almost always negative or barely minimal. Chilomonads were always negative but, when observed in food vacuoles, they were frequently stained, especially after quinine treatment (FIGURE 34). Cirrous ciliates, small and large, were almost always negative, faint reactions appearing occasionally

TABLE 3

	Food vacuoles				Cytoplasm			
	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>
Arsenilate								
2×10^{-4} M	-78	-100	-74	-70	-96	-100	-71	-81
10^{-3} M	-100	-100	-100	-100	-100	-100	-100	-100
Eserine								
10^{-6} M	-29	-13	-62	-30	-41	+14	-42	0
10^{-4} M	-18	+63	-29	-4	-36	+100	-42	+19
Fluoride								
2.5×10^{-4} M	-29	0	-17	-6	-54	0	+25	+46
2×10^{-3} M	-51	-100	-49	-28	-63	-100	-37	-4
Quinine								
5×10^{-3} M	+2	+88	-13	-22	-19	+100	+6	+15
2×10^{-2} M	+8	+13	-36	+12	-39	0	-23	+15
Taurocholate								
2×10^{-3} M	+8	0	-42	+24	-19	-29	-23	+27
2×10^{-2} M	-27*	+13	-42	-26	-51	-43	-33	-19
	-4†							

* Large vacuoles.

† Medium and small vacuoles.

after pretreatment with 2×10^{-2} M quinine; when observed in food vacuoles, they were almost always intensely stained even under conditions of inhibition of the cytoplasmic reactions (FIGURE 35). *Colpidium* was frequently negative, faint reactions appearing occasionally under all conditions and heightened somewhat by quinine. *Colpidium* observed in the food vacuole was much more intensely stained. Small euglenoids, infrequently observed on cover slips after processing, were negative. Large euglenoids were usually negative and occasionally minimal, although intensities were somewhat heightened after quinine. *Paramecium* intensities were moderate (FIGURE 37) reduced by arsenilate, fluoride, and higher concentrations of eserine and taurocholate, and heightened by quinine (FIGURE 36) and lower concentrations of taurocholate. *Paramecium*, when observed in the food vacuoles, was much more intensely stained. *Spirostomum* was almost always negative and barely affected even by quinine (FIGURE 38), yet in the food vacuole of *Pelomyxa* positive reactions were noted. Rotifers were infrequently observed on cover slips after processing, and these were always negative. Tiny amoeboid forms observed occasionally on cover slips prepared from *P. carolinensis* culture (FIGURE 39) were deeply colored, especially after some types of quinine treatment. It is of interest to compare the intensities and thicknesses (units engraved on the fine adjustment) of a tiny "amoeba" and of *Spirostomum* on the same cover slip (2×10^{-3} M fluoride treatment both prior to and during incubation).

	Intensity	Thickness
Tiny "amoeba"	4.0	0.8
<i>Spirostomum</i>	0.0	3.5

Deposition in the small amoeba was of so fine a grain that resolution was not possible at 580X. Despite the greater thickness of *Spirostomum* (several

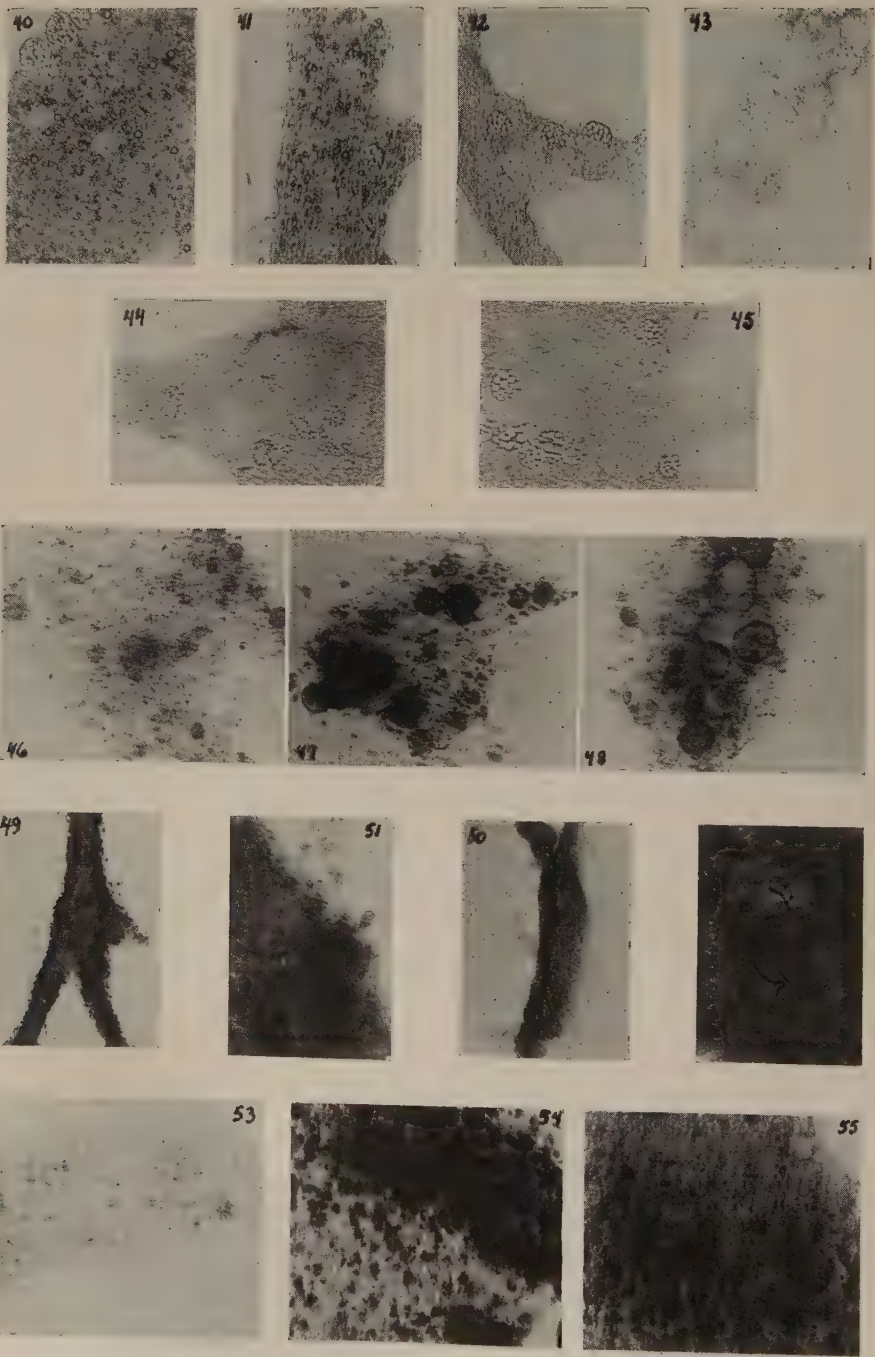
times thicker still if the thinnest portion of the small "amoeba" still showing coloration were considered), coloration in *Spirostomum* was negative. The annelid *Aeolosoma* was weakly colored, with specific coloration confined mainly to peristomal, prostomal, and pharyngeal regions (FIGURE 36). Spores were always negative. Debris, consisting of granular and amorphous material in which were embedded bacteria, monads and, occasionally, larger organisms poorly defined in the mass after processing, was usually negative, although minimal reactions were occasionally observed.

The intensities observed in the food vacuoles and cytoplasm of *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis* were too great to be accounted for by bacteria, debris, or food organisms present in their cultures.

All of the arguments presented at the conclusions of the beta-naphthyl acetate section in favor of sharp localization and against diffusion are recapitulated in the case of naphthol AS acetate.

Polyoxyethylene sorbitan monostearate (Tween 60). Microscopic enzyme chemistry, as it applies to this ester, depends on the precipitation of calcium stearate as a result of specific activity toward the soluble monostearate. Following cation exchange with lead and anion exchange with sulfur, sites of activity are visualized as lead sulfide. Endogenous cation such as calcium and basic protein and lipid groups must therefore be accounted for and, where possible, eliminated before incubation and cation exchange with lead. Demineralization has been discussed under *Materials and Methods*, in which the figures referred to *A. dubia*. Additional explanatory text accompanies FIGURES 40 to 45 that refer to *A. proteus*. Cation capable of exchanging with lead was concentrated in the refractile bodies, but present also in the cytoplasm. The presence of calcium and of other metallic elements in these sites has been described (Heller and Kopac, 1956). Elimination of cation from the refractile bodies appeared to be associated with the disappearance of these bodies and the concomitant appearance of additional cation in the cytoplasm, provided the washing following citrate or Versenate was very brief. The fact that solubilization of endogenous calcium and/or magnesium even by chelation must be followed by removal of the complex before immersion in lead is in accord with both the greater complexing affinity of Versene for lead and the degree of affinity of sulfur for lead that enable it to release lead even from the chelate (Bersworth, 1952). The elimination of nonspecific soluble calcium absorbed from the incubation mixture is dealt with in the section on postincubation washing. The elimination of nonspecific soluble cation, which is part of the cation exchange medium, is dealt with in the section on postcation exchange washing. Neither elimination was troublesome.

Validation for lead sulfide as an end product of specific activity was sought by means of variation in incubation times, by the use of general inactivation procedures involving heat, iodine, and phenol pretreatments, and by the use of various types of deficiency media. Cobalt, which has an inhibiting effect on lipase, for which reason its use in the Tween technique is forbidden (Gomori, 1952a), was employed in some cases in the place of calcium in the incubation medium. In some cases, incubation with cobalt was followed by direct anion exchange with sulfur as in the myristoyl choline technique. In some cases such



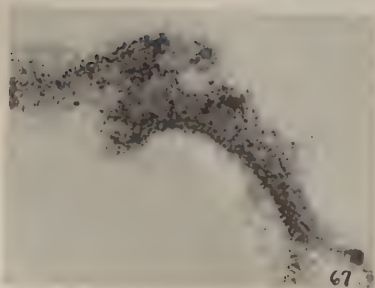
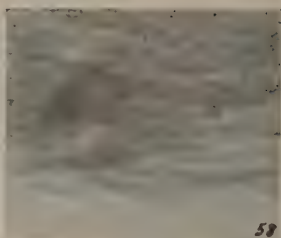
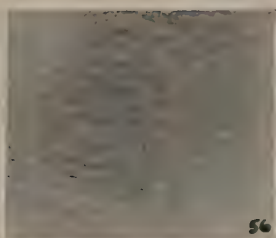
incubation was followed by cation exchange with lead and anion exchange with sulfur, as would be normal following incubation in medium containing calcium.

Compared with 24 hours of incubation, food vacuole and cytoplasm intensities in all species were greatly increased after 48 hours; with 72 hours of incubation, in all cases except in *A. proteus*, the intensities generally declined, especially in the food vacuoles (FIGURES 46 to 48). Nuclear staining was not observed. The nuclei, when recognizable, appeared as light hyaline bodies.

Three possibilities accounting for the decline in intensity with prolonged incubation may be considered: (1) resolubilization of calcium stearate; (2) resynthesis of substrate; and (3) inefficient ion exchange with lead or with sulfur or with both. The first seems unlikely because of the low degree of solubility of the specific deposits, because *A. proteus* intensities continued to increase to 72 hours in the same medium and because pallor first appeared in the center of the food vacuole (FIGURE 48). Resynthesis of substrate attending excessive accumulation of the product seems unlikely because of the solubility principle and because the periphery of the pale vacuole was quite dark (FIGURE 48). Inefficient ion exchange was strongly supported by the internal location of the pallor in the food vacuole, by the dark periphery, by increasing intensity in *A. proteus* in which the intensity after 48 hours had lagged behind *A. dubia*, and by the fact that a medium containing a tenfold concentration of calcium resulted in food vacuoles simulating prolonged incubation (FIGURES 49 to 52). Indeed, it is probably for this reason that Gomori recommends replacement of calcium by lead at elevated temperatures. The recommendation of Pearse was followed instead because of the greater losses of animals from cover slips under Gomori conditions. In passing, it will be noted from FIGURE 50 that general inactivation procedures, in this case with phenol, did not abolish coloration entirely; for this reason, both gross and net color wheel intensities obtained by subtracting certain basal intensities were recorded.

Cytoplasmic deposition was inseparably fine and coarse in *A. dubia* (FIGURE 47), fine and light with sparse coarse sprinkling in *A. proteus* (FIGURE 53), and fine to heavily coarse in *P. carolinensis* (FIGURE 54) and *P. illinoisensis* (FIGURE 55). The two types of deposition in the cytoplasm, the coarse deposition more intense and behaving differently from the fine under different conditions, made

←
FIGURES 40 to 45. Endogenous cation in *A. proteus* capable of exchange with lead with incubation by-passed. $\times 290$. (40) Sixteen minutes in water prior to lead and sulfide. (41) Twenty minutes in water prior to lead and sulfide. (42) Fifteen minutes in citrate buffer and 1 minute in water. (43) Fifteen minutes in citrate buffer and 5 minutes in water. (44) Fifteen minutes in Versene and 1 minute in water. (45) Fifteen minutes in Versene and 5 minutes in water. FIGURES 46 to 48. Progression and regression of specific deposition in *A. dubia* with Tween 60 with increasing incubation time. $\times 290$. FIGURES 49 and 50 $\times 70$ and 51 and 52 $\times 290$. *P. carolinensis*. Regression of specific deposition with prolonged incubation is simulated by increased cation in the medium, supporting the concept of inefficient ion exchanges. (49) Normal calcium. (50) Phenol inactivation; basal levels of nonspecific coloration are troublesome in large cells processed by the Gomori procedure. FIGURE 51. Normal calcium concentration $\times 0.1$. FIGURE 52. Normal calcium concentration $\times 10.0$; food vacuoles show pallor, as with prolonged incubation. FIGURES 53 to 55. Normal reactions in *A. proteus*, *P. carolinensis*, and *P. illinoisensis*, respectively, with Tween 60; coarse deposition is heavy in the pelomyxae; the reactions in *A. proteus* are exceptionally weak. Deposition in nuclei not observed. $\times 290$.



it necessary to record the two types of cytoplasmic deposition separately. The species in decreasing order of net food vacuole intensities were *A. dubia*, *P. carolinensis*, *P. illinoisensis*, and *A. proteus*. The species in decreasing order of net intensities for the relatively fine cytoplasm deposition were *A. dubia*, *P. illinoisensis*, *P. carolinensis*, and *A. proteus*, with *A. dubia* intensities possibly overrated because of difficulty in separating the two types of cytoplasm intensities. The species in decreasing order of intensities for the coarse cytoplasm deposition were *Pelomyxa*, *A. dubia*, and *A. proteus*.

Pretreatment with boiling water (FIGURE 56) or phenol (FIGURE 58) completely eliminated the coarse cytoplasm intensities, although levels of coloration remained. Some residual levels after iodine pretreatment (FIGURE 57) were greater than after heat or phenol, due probably to residual activity, for the same thing was noted with the naphtholic substrates. Treatment with 25 per cent sodium thiosulfate had little effect on the residual coloration, indicating that iodine staining was not responsible. Residual levels were somewhat higher after heat pretreatment than after phenol pretreatment, suggesting either somewhat less inactivation by heat or some increase in nonspecification binding as a result of heat: probably the latter, for with the naphtholic substrates with which the specific and nonspecific colorations are of very different hue, it was after phenol pretreatment rather than after heat pretreatment that slight residual levels remained.

There was little difference between either food vacuole or fine cytoplasm residual levels after incubation in deficiency media lacking substrate and deficiency media lacking both substrate and calcium (FIGURES 59 and 60), suggesting that nonspecific calcium binding was of small import. Residual levels after incubation in the absence of both calcium and substrate would appear then to be due to nonspecific lead binding superimposed on a minimal endogenous coloration inherent in the animals themselves, an endogenous coloration that could be observed both in the living state and, heightened somewhat, in animals after fixation. The substitution of cobalt for calcium followed by direct sulfiding without intermediate passage through lead (FIGURE 61) resulted in the absence of coarse deposition and in food vacuole and fine cyto-

← FIGURES 56 to 58. General inactivation procedures prior to incubation with Tween 60; boiling water, iodine, and phenol, respectively. The large *P. carolinensis* cells appear to make it easier to recognize that heat may alter cytoplasmic entities in favor of cation binding and that iodine may leave some residual activity in the food vacuole (as was noted also with betanaphthyl acetate) $\times 290$. FIGURES 59 to 64. Alterations in media in the Tween 60 technique. $\times 290$. FIGURE 59. *P. illinoisensis*. Substrate omitted. FIGURE 60. *P. illinoisensis*. Both substrate and calcium omitted. FIGURE 61. *P. carolinensis*. Cobalt substituted for calcium. Incubation followed by direct sulfiding as for myristoyl choline. Cobalt appears to be inhibiting, as is typical for lipase. FIGURE 62. *P. illinoisensis*. Cobalt substituted for calcium. Incubation followed by lead and sulfide. FIGURE 63. *P. carolinensis*. Calcium omitted. The liberated stearic acid is capable of depositing in the absence of precipitating calcium and of combining with lead subsequent to incubation. FIGURE 64. *P. carolinensis*. Calcium greatly reduced. Loosely textured crystals, often with radiating needles. FIGURES 65 and 66. *P. carolinensis*. Inhibiting effects of low and high concentrations of quinine on reactions with Tween 60. $\times 290$. FIGURES 67 and 68. *P. carolinensis*. Compared with the normal Tween 60 reaction, taurocholate may intensify the reactions in food vacuoles and coarse deposition. $\times 70$.

plasm intensities lower even than those following inactivation or incubation in deficiency media where passage through lead was included. This suggested that the nonspecific binding of lead was indeed a significant part of the nonspecific basal level, and it suggested also that the enzyme, reacting with the monostearate, might be separate from the enzyme reacting with myristoyl choline, with which cobalt and direct sulfiding resulted, as will be seen, in intense cobalt sulfide deposition. The substitution of cobalt for calcium, followed by cation exchange with lead before sulfiding (FIGURE 62), resulted in iodine-inhibitionlike intensities with occasional sparse sprinkling of aggregates finer, however, than the coarse deposition with normal incubation.

A feature that was surprising at first thought was the occurrence of a coarse deposition, although scant, inflorescent, and generally of low intensity, after incubation with deficiency media containing substrate but lacking precipitating calcium (FIGURE 63); in addition, the fine cytoplasm deposition was slightly more intense and the food vacuole deposition was considerably more intense than when both substrate and precipitating cation were absent; in the absence of precipitating cation, then, a phenomenon analogous to azo dye postcoupling appeared to have taken place as the result of a degree of stearic acid insolubility sufficient for stearic acid trapping and subsequent conversion to lead stearate. Reduced calcium, approximately 5 per cent of normal, was capable of producing coarse cytoplasm deposition (although still extremely loose-textured and of large crystal size), food vacuole deposition considerably more intense (often with radiating needles), and fine cytoplasm deposition somewhat more intense than similar types of deposition in the presence of substrate but with calcium entirely absent (FIGURE 64).

The difficulty with the "Gomori rationale," then, was the persistence of several levels of coloration, even after demineralization procedures, due in part to endogenous coloration, nonspecific cation binding and, possibly, nonenzymatic or even bacterial breakdown of substrate. The most logical basal level for transformation of gross intensities to net intensities was considered to be one integrating all of the nonspecific levels. Such a basal level might be expected to result from incubation in normal medium followed by normal processing, provided enzyme activity was nil. Closest to meeting these requirements were the heat and phenol inactivation levels which, moreover, were quite similar to each other in any given species. These basal levels tended to be rather high, raising the question of whether or not such pretreatments resulted in alterations in favor of heightened intensities (quinine treatments resulted in intensities lower even than heat or phenol inactivation levels). Such effects would result in net intensities too low and, with them, an exaggeration of the effects of the various chemical agents would occur; but it was felt all the more that, if this were so, the net intensities then could be attributed to specific enzyme activity. Other basal levels such as those after the use of deficiency media lacking substrate would fail to record basal levels that might result from nonspecific breakdown or from bacterial breakdown of substrate; such media would fail, also, to affect the basal levels in the opposite direction because of what appeared to be a leaching effect on the part of the Tween substrates, as will be shown in the section dealing with the monooleate ester. Deficiency

media containing substrate, but lacking calcium, would record basal levels that are too high because, included, are specific intensities due to trapping of insoluble fatty acid even without cationic co-precipitation. Using, then, the intensities after heat or after phenol, whichever were highest, and subtracting these from all other gross intensities, by far the greatest net intensities were for the coarse cytoplasm deposition, intermediate intensities were for the food vacuoles; the lowest intensities were for the fine cytoplasm deposition.

The coarse deposition resembled crystals of the mother substrate, polyoxyethylene sorbitan monostearate, which exists as a solid in equilibrium with the liquid phase at room temperature. This crystal form was assumed, apparently, by calcium stearate when deposited as a result of intense activity and reproduced in faithful replica as lead sulfide through the ensuing exchanges with lead and sulfur. Some combination of factors, such as loci of more intense activity, concentration of loci of activity, and loci of more specific activity toward the monostearate ester, is suggested to account for their formation.

The percentages of change from normal of the numerical averages of the food vacuole intensities and of the fine and coarse cytoplasmic intensities in the four species of amoebae, following treatment with chemicals both prior to and during incubation, are shown in TABLE 4.

The most noteworthy effects appeared to be the very considerable inhibition by quinine (FIGURES 65 and 66) and activating effects, at times, on the food vacuoles and coarse deposition by the lower concentration of taurocholate.

Treatment with chemicals during incubation only produced much the same results, except that somewhat more activation with the lower concentration of taurocholate was observed (compare FIGURE 67 with FIGURE 68), and occasional activation was obtained with the higher concentration (TABLE 5).

The effects of treatment with chemicals prior to incubation only were less marked; a notable feature was the appearance of activated reactions even with the higher concentration of taurocholate (TABLE 6).

Chilomonads from cultures (FIGURE 69) or in food vacuoles were without net intensities except occasionally under the influence of taurocholate. Cirriform ciliates from culture (FIGURE 70) were also negative or occasionally minimal when treated with taurocholate but, when observed in food vacuoles, were frequently quite intense (FIGURE 71). *Colpidium* was negative except for vacuolelike bodies that occasionally showed positive net intensities under the influence of taurocholate (FIGURE 72); in amoeba food vacuoles, colpidia intensities were often positive. Euglenoids (FIGURE 73) were of low level intensities, the intensities being occasionally heightened by taurocholate; in food vacuoles, the intensities were somewhat higher (FIGURE 74). *Paramecium* occasionally approached intensities observed in *Amoeba* and *Pelomyxa* food vacuoles. *Spirostomum* intensities approached those of *Paramecium*, but contained in addition more intense vacuolelike bodies. Rotifers were of low level intensity and frequently contained spots of somewhat higher intensity, but even these were normally at phenol inactivation levels (FIGURE 75), except occasionally with taurocholate (FIGURE 73).

Aelosoma feeding on bottom debris was of low level body-wall intensity (FIGURE 76), generally at inactivation levels or lower, hence negative. The

TABLE 4

	Food vacuoles				Cytoplasm, fine				Cytoplasm, coarse			
	A.d.	A.p.	P.c.	P.i.	A.d.	A.p.	P.c.	P.i.	A.d.	A.p.	P.c.	P.i.
Arsenilate 2×10^{-4} M 10^{-3} M	-79	-70	-20	-67	-73	-100	0	-100	-100	(a)	+14	(b)
	-38	-60	-16	-38	-87	-100	-11	-100	(c)	(a)	0	-30
Eserine 10^{-6} M	-84	-30	-48	-42	-100	-100	+67	-100	(d)	-20	0	(e)
Fluoride 2×10^{-3} M	(f)		(f)		(f)		(f)		(g)		(h)	
Quinine 5×10^{-3} M	-89	-30	-68	-25	-100	-100	-100	-100	(a)	(a)	-18	(i)
2×10^{-2} M	-100	-100	-100	-100	-100	-100	-100	-100	-100	-100	-100	-100
Taurocholate 2×10^{-3} M	-11	-30	+32	+4(j)	-100	-100	-100	-100	(k)	(a)	+23	-14
2×10^{-2} M	+14	-100	-68	-100	-100	-100	-100	-100	+40(l)	-100	-100	-100

- (a) Coarse deposition almost entirely eliminated with intensity of few deposits, -20.
(b) Coarse deposition almost entirely eliminated with intensity of few deposits, -45.
(c) No coarse deposition; instead, small aggregates appearing to lie in vacuoles, -20.
(d) No coarse deposition; instead, many small aggregates appearing to lie in vacuoles, -0 to -20.
(e) Coarse deposition almost entirely eliminated with intensity of few deposits, -6.
(f) Incubation in the absence of calcium but in the presence of fluoride resulted in reduced intensities compared to incubation in the absence of calcium but without fluoride.
(g) Incubation in the absence of calcium but in the presence of fluoride resulted in intensities similar to incubation in the absence of calcium but without fluoride.
(h) Incubation in the absence of calcium but in the presence of fluoride resulted in -100 intensities compared to incubation in the absence of calcium but without fluoride.
(i) Coarse deposition almost entirely eliminated with intensity of few deposits, -17.
(j) In portions of some food vacuoles.
(k) No coarse deposition; instead, many tiny to large aggregates appearing to lie in vacuoles, +20.
(l) Coarse deposition to +40 and very many tiny aggregates appearing to lie in vacuoles, +40.

TABLE 5

	Food vacuoles				Cytoplasm, fine				Cytoplasm, coarse			
	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>
Quinine												
5 × 10 ⁻³ M	-61	-70	-8	-67	-100	-100	-100	-100	(a)	(a)	+8	(b)
2 × 10 ⁻² M	-93	-20	-84	-67	-100	-100	-100	-100	-100	-100	-100	-100
Taurocholate												
2 × 10 ⁻³ M	-71	+40	+52	+79	-100	-100	+11	-7	(c)	(a)	+34	+22
2 × 10 ⁻² M	-7	-100	-60	-100	-100	-100	-100	-100	+20(d)	-100	-86	-100

(a) Coarse deposition almost entirely eliminated with intensity of few deposits, -20.

(b) Coarse deposition almost entirely eliminated with intensity of few small deposits, -36.

(c) No coarse deposition; instead, very many small aggregates appearing to lie in very small vacuoles with losses in intensity from 0 to -20.

(d) In addition to coarse deposition showing gains in intensity, many small-to-large aggregates appearing to lie in vacuoles also show these gains in intensity.

TABLE 6

	Food vacuoles				Cytoplasm, fine				Cytoplasm, coarse			
	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>
Taurocholate												
2 × 10 ⁻² M	-39	-20	-8	+25(a)	-90	+75	+67	+43(b)	0	-20(c)	-8	+14(d)

(a) In some food vacuoles.

(b) In some areas.

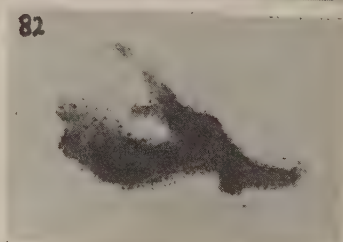
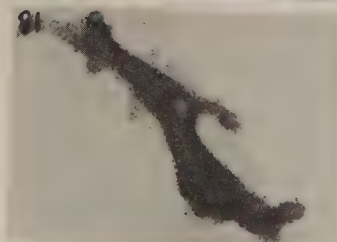
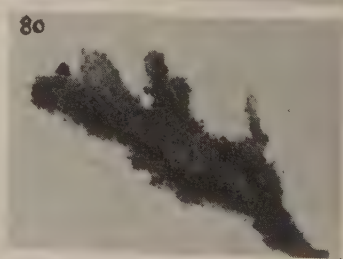
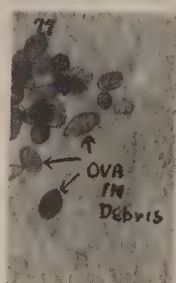
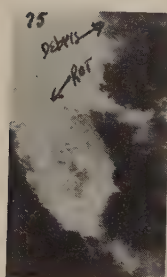
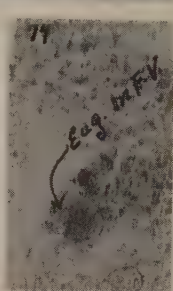
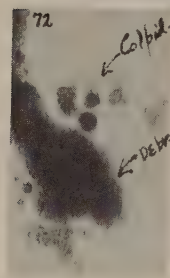
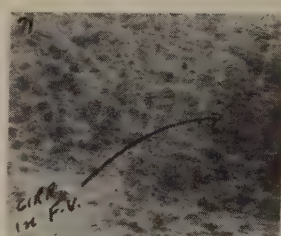
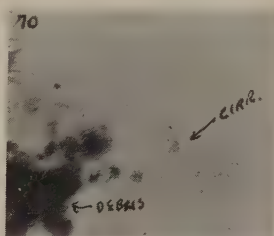
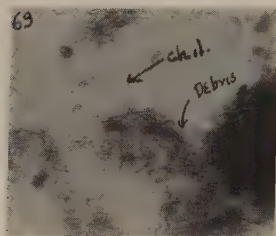
(c) While intensity of coarse deposition is reduced -20, many small aggregates appear to be lying in vacuoles with an increase in intensity of +20.

(d) Scattered.

middle third of the annelid contains an expansion of the gut in which, by contrast, deposition was strikingly intense and coarse. Anterior to this expansion, deposition was rarely observed, while in the posterior third of the gut deposition was more often observed, but almost always of an intensity little exceeding that of the body wall.

Since *Aeolosoma* was observed to be a voracious bottom-debris feeder, it was of interest to compare the reactions obtained in the gut expansion with those of the coarse deposition in *P. illinoisensis* and of bottom debris, all from the same culture bowl (TABLE 7). There appeared to be insufficient correlation between the effects of the differentiating agents mentioned in TABLE 7 on the three types of coarse deposition, especially on the type of coarse deposition in *P. illinoisensis*; inactivation procedures pointed to the deposition in *Pelomyxa* and in *Aeolosoma* as having been enzymatically mediated in good part, whereas very strong intensities remained in the debris after such inactivation procedures (except after heat). In the debris, absence of coarse deposition in the absence of substrate and absence with cobalt substitution for calcium followed by direct sulfiding, as in the myristoyl choline technique, suggested mediation, in the debris reactions, of thermolabile but relatively phenol- and iodine-stable enzymes, presumably bacterial for the most part.

In debris from *A. proteus* cultures, an additional complication existed in the form of ova. Ova were observed on cover slips after iodine, diminished cal-



cium, absence of substrate (FIGURE 77), and treatment with a variety of diagnostic chemicals. Wherever they were so observed, they were of great intensity. After treatment with heat the intensities were weakened in some of the ova, so that a greater range of intensities resulted. However, the intensities of some were remarkably high for this type of inactivation, making it appear that little if any of the intensities in the ova were enzymatic.

However, it appeared that a high degree of endogenous coloration or of nonspecific cation binding material, leached in some cases by boiling water, was present. Debris reactions could not be made to account for the food vacuole reactions, either. *Aeolosoma*, obviously a debris feeder, possessed coarse deposition, in the expanded portion of the gut, very similar in appearance to the coarse deposition in the debris, whereas *Amoeba* and *Pelomyxa* food vacuoles were of entirely different appearance and, even when the food organisms were not identifiable, were of sharp outline with contents compactly homogeneous, variably granular, or filled with round bodies, such as are present in chilomonads and larger euglenoids. It should be pointed out, however, that even in *Aeolosoma*, contradictions appear if attempts are made to relate intensities or the effects of chemical agents either on the intensities or on the character of the coarse deposition, although this may be due to contributions of enzyme on the part of the worm on the one hand and an opposing tendency for enzyme in the debris to be destroyed in alimentation as shown by the fact that, posterior to the expansion of the gut, the intensities dwindle abruptly and the deposition becomes greatly reduced in coarseness. Debris reactions could not be made to account for the food vacuole reactions for another reason, for debris taken in with a food organism would be expected to form a ring around the food organism, as was actually observed in several instances (FIGURE 78).

Debris reactions could not be made to account for the food vacuole reactions also because of notable contradictions if attempts were made to relate the actual intensities of, or the effects of chemicals on, the debris on the one hand and the food vacuoles on the other.

Another line of evidence for the relation between debris reactions and reactions in amoebae derives from the use of antibiotics (1000 units of penicillin G sodium per milliliter).

Pelomyxa demonstrated high physiological activity in the presence of the antibiotic, judging from their proliferative capacity. The initial and final

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FIGURE 69. Poor reactions in chilomonads with Tween 60. $\times 290$. FIGURE 70. Poor reactions in cirrous ciliates with Tween 60. $\times 290$. FIGURE 71. Cirrous ciliate in food vacuole more darkly stained. $\times 290$. FIGURE 72. Poor reactions in *Colpidium* except for included vacuoles. $\times 290$. FIGURE 73. Poor reactions in euglenoids with Tween 60. $\times 290$. FIGURE 74. Euglenoids in food vacuole more darkly stained. $\times 290$. FIGURE 75. Large poorly reacting rotifer in highly active debris. $\times 290$. FIGURE 76. *Aeolosoma*. Body-wall reactions with Tween 60, poor; striking intensities in gut expansion. $\times 70$. FIGURE 77. Ova observed only in *A. proteus* culture; nonspecific coloration in absence of substrate unusually intense. $\times 290$. FIGURE 78. Probable extent of contribution of debris to reactions in the amoebae. See also FIGURES 98 and 99. $\times 290$. FIGURES 79 to 82. *P. carolinensis* in debris-free culture fluid 24 hours, in the same + penicillin 24 hours, in the culture fluid 72 hours and in the same + penicillin 72 hours; reactions with Tween 60 remain intense and retain coarse component. $\times 70$.

TABLE 7

	<i>Pelomyxa</i>	<i>Aeolosoma</i>	Debris
Normal	6.4	7	9
Heat	0.0	4 rare	4
Iodine	0.0	5 few	9
Phenol	0.0	5 few	9
No calcium-no substrate	0.0	3	3
Reduced calcium	4.6 few	7	7 to 8
No substrate	0.0	3 rare	0
Cobalt to cobalt sulfide	0.0	3 to 4	0
Cobalt to lead to lead sulfide	4.4 few	5	9
Arsenilate (2×10^{-4} M)			
During incubation	4.8	6	9
Prior to and during incubation	3.5 few	6 to 7	8
Arsenilate (10^{-3} M)			
Prior to incubation	5.2	8	9
During incubation	6.2	—	8
Prior to and during incubation	4.6	6	7
Eserine (10^{-5} M)			
Prior to incubation	6.4	6	6 to 7
During incubation	5.6	9	9 to 10
Prior to and during incubation	6.0 few	—	8
Fluoride (2×10^{-3} M)			
Prior to incubation	5.7 small	8	8
Quinine (5×10^{-3} M)			
During incubation	4.1 small	—	—
Prior to and during incubation	5.3 few	4 to 5	8
Quinine (2×10^{-2} M)			
Prior to incubation	4.5 small	5	9
During incubation	0.0	4 to 5	9 to 10
Prior to and during incubation	0.0	4 to 6	10
Taurocholate (2×10^{-3} M)			
During incubation	7.8	9 to 10	9 to 10
Prior to and during incubation	5.5 few	10 few	10
Taurocholate (2×10^{-2} M)			
Prior to incubation	7.3 few	7 to 8 (influorescent)	10
During incubation	0.0	8 few	9
Prior to and during incubation	0.0	—	6 to 10

numbers in the dishes one, two, and three days old are shown in TABLE 8. In all animals under all conditions, the cytoplasm was filled with a coarse deposition that graded down imperceptibly to one of fine dimensions (FIGURES 79 to 82) following incubation with the stearate ester.

Finally, some interesting insights were gained from "*pH*-activity" curves and the effects on these of taurocholate. A study on *pH*-activity curves in a rat pancreatic lipase-olive oil system and the effects on these of different concentrations of taurocholate (Börgerstrom, 1954) seemed to provide an explanation for the apparent activation of lipases in general and of lipase-Tween systems in particular.

Current explanations based on emulsification or solubilization of lipids would not appear to be tenable with the already soluble Tweens. The apparent activation (or inhibition) at any given *pH* could be explained on the basis of displacement of the *pH*-activity curves and their optima by different concentra-

TABLE 8

	Numbers of <i>Pelomyxa</i>	
	Initial	Final
Dishes one day old		
Culture fluid	5	6
Culture fluid + penicillin	5	5
Brandwein's solution (fifth passage)	5	5
Dishes two days old		
Culture fluid	5	12
Culture fluid + penicillin	5	8
Brandwein's solution (fifth passage)	5	12
Dishes three days old		
Culture fluid	5	17
Culture fluid + penicillin	5	16
Brandwein's solution (fifth passage)	5	12

tions of taurocholate, an explanation tenable as well with the soluble Tweens. The importance of this displacement is emphasized by the fact that, even with olive oil under "test-tube" biochemical conditions, no maximum with taurocholate, in any concentration and at any *pH*, exceeded the maximum in the absence of taurocholate. Three of the taurocholate conditions in the Börgstrom curves happen to have been standard procedure in the complete experiments with the Tweens: incubation in the absence of taurocholate, incubation in the presence of the lower concentration of taurocholate (2×10^{-3} M represents 107 mg. per cent or the equivalent of Börgstrom's 0.1 per cent concentration) and incubation in the presence of the higher concentration of taurocholate (2×10^{-2} M represents 1070 mg. per cent or the equivalent of Börgstrom's 1.0 per cent concentration). In a general way, the presence of taurocholate in the incubation medium did result more often in activation at the lower concentration than at the higher, as might be expected if the amoeba enzymes were similar to rat pancreatic lipase, for the buffer was set at *pH* 7.3, at which *pH* Börgstrom's curves show that 0.1 per cent would tend to be somewhat activating and 1.0 per cent somewhat inhibiting. Börgstrom's curves reveal also that at this *pH* these differences tend to be of small order, accounting perhaps for the lack of clear-cut activation or inhibition in all cases in the amoebae.

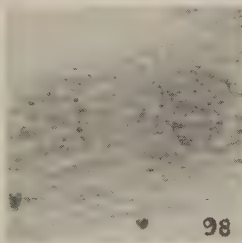
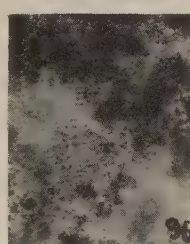
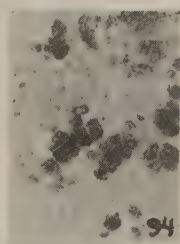
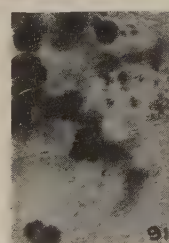
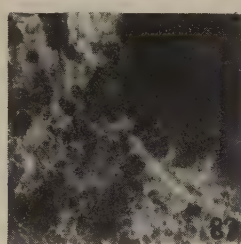
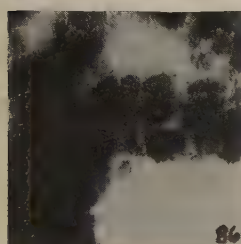
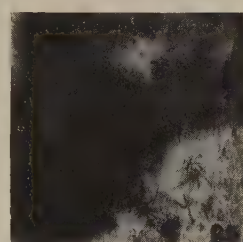
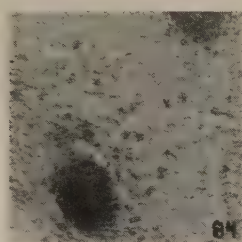
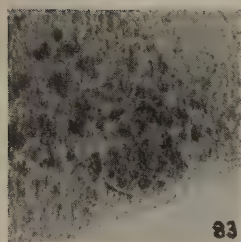
Since the microscopic enzyme technique involves not only enzyme activity, but critical requirements for prompt precipitation at enzyme sites which might be affected by *pH*, it is only in passing that mention is made of the *pH*-activity reactions in *P. carolinensis*. The tris hydroxymethyl amino methane buffer was titrated with hydrochloric acid to *pH* 5.1, 7.0, and 9.0 or not treated with acid (*pH* 10.5). The *pH* at the end of the incubation period (24 hours) was considered the effective *pH*. Food vacuole maxima were obtained in the *pH* region of 8.5; these were shifted by the low concentration of taurocholate to the region of *pH* 7.0. With the higher concentration of taurocholate, extremely low levels of activity were obtained at all *pH* values, but there was

evidence of pending intersection in the region of pH 10, beyond which apparent activation might have been obtained with the higher concentration of taurocholate. Coarse deposition in the cytoplasm was maximum in the region of pH 7.0, and most of the coarse deposition showed a somewhat lower maximum in the same pH region with the low concentration of taurocholate, but occasional deposits with this concentration of taurocholate were more intense below and above the pH regions 6 and 8, respectively. No coarse deposition was noted with the higher concentration of taurocholate in this experiment. Fine deposition in the cytoplasm showed a pattern somewhat similar to the coarse deposition except that no apparent activation was noted in this experiment with the low concentration, except above pH 9. The lowest intensities were noted with the high concentration of taurocholate, except in the region of pH 9 where the curve intersected and rose above both the low taurocholate and zero taurocholate curves. Debris reactions contrasted markedly with those in *Pelomyxa*. In the absence of taurocholate, *Pelomyxa* reactions, especially in the cytoplasm, were low at extremes of pH (FIGURES 83 and 84); debris reactions were not only more intense than any in *Pelomyxa*, but maintained the higher intensity levels at all values (FIGURES 85 to 87). This is not surprising in view of the fact that the debris probably represented a number of different species of microorganisms, each with its enzyme's or enzymes' own pH optimum or optima. Another striking effect was the apparent activation of debris reactions by both low and high concentrations of taurocholate at all pH levels (FIGURES 88 to 93).

The differences between the behavior of the debris reactions and those in *Pelomyxa* support the view that debris reactions contributed little to the enzyme reactions observed in the amoebae. It remains to draw on a final line of evidence, perhaps the most striking of all. Debris reactions were obtained in the absence of substrate if taurocholate were present in the medium at all pH levels (FIGURES 94 to 96); the deposition in *Pelomyxa* under the same conditions was confined to occasional food vacuole stippling or to a crescent between an ingested organism and the food vacuole wall (FIGURES 97 to 99). This type of deposition may represent the full extent to which debris reactions contributed to the reactions in the amoebae. Heightened over-all coloration of the cytoplasm was noted with taurocholate in the absence of substrate at an ele-

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FIGURES 83 and 84. Reduced *P. carolinensis* reactions with Tween 60 at pH levels lower (pH 5.0) than and higher (pH 8.5) than normal. $\times 290$. FIGURES 85 to 87. Debris reactions with Tween 60 are not only more intense than in *Pelomyxa* but a plateau is maintained throughout the range tested; figures shown are for pH 5.0, 7.0, and 9.5, respectively. $\times 290$. FIGURES 88 to 93. Debris reactions with Tween 60 in the presence of both low and high concentrations of taurocholate are unusually intense; the intensities are high throughout the pH range; the figures shown are respectively for low taurocholate, pH 5.0; low taurocholate, pH 7.0; low taurocholate, pH 8.5; high taurocholate, pH 7.0; high taurocholate, pH 8.0; high taurocholate, pH 9.5. $\times 290$. FIGURES 94 to 96. Some of the above intensities are contributed by taurocholate, this acid presumably acting as a substrate; the figures shown are for the low taurocholate concentration in the absence of Tween 60, pH 5.0; pH 7.0; pH 9.0. $\times 290$. FIGURES 97 to 99. Such reactions are never observed in *Pelomyxa* except as stippling in the food vacuole or confined to a space between the food organism and the food vacuole wall. The conditions figured are respectively low concentration of taurocholate in the absence of Tween 60, pH 5.0; pH 7.0; pH 9.0. This may represent the full extent of the contribution of the debris reactions to the reactions in the amoebae. $\times 290$.



vated pH, but this was not of concern, since the investigation of carboxylic esterases was not carried out at this pH. The possible mechanism of deposition with taurocholate in the absence of substrate is given in the discussion section.

Polyoxyethylene Sorbitan Monolaurate (Tween 20)

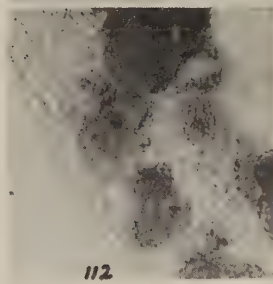
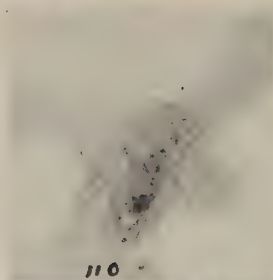
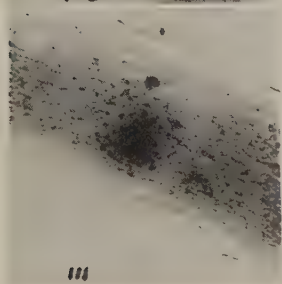
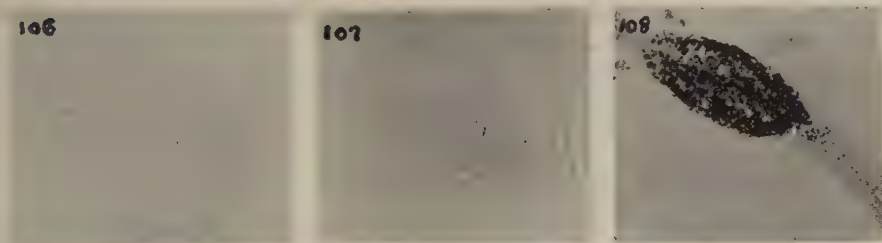
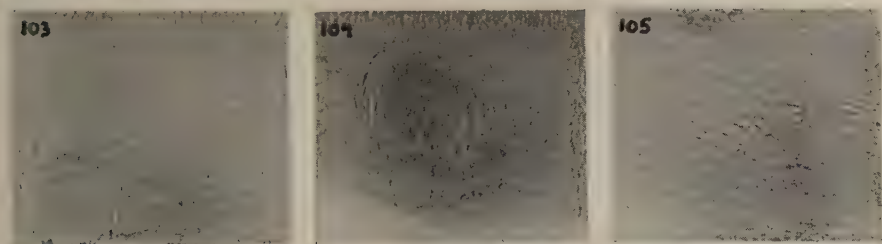
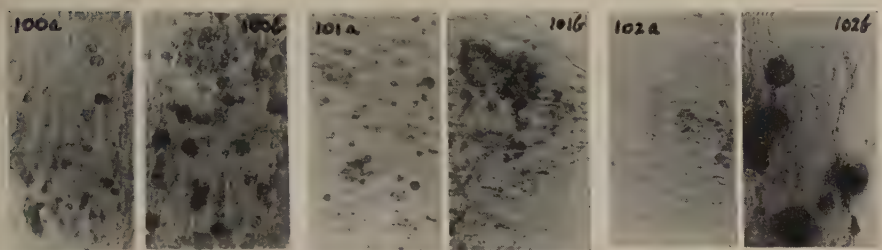
In preliminary experiments, poor microscopic enzyme chemical reactions were obtained with this ester.

Polyoxyethylene Sorbitan Monooleate (Tween 80)

Microscopic enzyme chemistry, as it applies to this ester, depends on the precipitation of calcium oleate as a result of specific activity toward the soluble monooleate. Following cation exchange with lead and anion exchange with sulfur, sites of activity are visualized as lead sulfide. Endogenous cation such as calcium and basic protein and lipid groups must therefore be accounted for and preferably eliminated before cation exchange. This has been dealt with in the previous section, as have the validating procedures and procedures for transforming gross intensities to net intensities.

Nuclear staining was not observed; the nuclei, when recognizable, appeared as light hyaline bodies. Cytoplasmic deposition consisted of both fine and coarse types, but the coarse deposition was in the form of disks instead of brush-heaps, as with the previous ester. Although fairly numerous in the *Pelomyxa* after normal incubation, these forms were scant in *A. dubia* and tended to be absent in *A. proteus*. Net intensities tended to be of small dimension (in terms of units on the color comparator wheel) in the food vacuoles and especially small in the fine cytoplasm deposition. The species in decreasing order of net food vacuole intensities were: *P. illinoisensis*, *P. carolinensis*, and *A. dubia*; the food vacuole intensities in the last two being of approximately equal magnitude. *A. proteus* food vacuoles were rarely dark, and a complication existed in the form of ingested ova that were held to be responsible for occasional food vacuole intensities higher than in all of the other species. The species in decreasing order of net intensities for the relatively fine cytoplasm deposition were *P. carolinensis*, *P. illinoisensis*, *A. dubia*, and *A. proteus*. The species in decreasing order of net intensities for the coarse cytoplasm deposition were *P. carolinensis* (FIGURE 100), *P. illinoisensis* (FIGURE 101), *A. dubia* (FIGURE 102)—rare to none—and *A. proteus* (none).

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FIGURES 100a to 102a. Reactions in *P. carolinensis*, *P. illinoisensis*, and *A. dubia*, respectively, with Tween 80; reaction in *A. proteus* was negligible. Reactions in all species, except for coarse deposition, were weaker with Tween 80 than with other esters in the Gomori classification. ×290. FIGURES 100b to 102b. Reactions with 50 per cent increase in incubation time; reaction in *A. proteus* negligible even with 100 per cent increase in time. ×290. FIGURES 103 and 104. General inactivation procedures prior to incubation with Tween 80, boiling water, and iodine, respectively (*P. carolinensis*). ×290. FIGURES 105 and 106. Deficiency media in Tween 80 technique. ×290. (105) *P. carolinensis*, calcium omitted. (106) *P. illinoisensis*, calcium reduced. FIGURE 107. Rotifer reactions negligible with Tween 80. ×290. FIGURE 108. *Aeolosoma* reactions with Tween 80; observations as for FIGURE 76. ×70. FIGURES 109 to 112. Reactions with myristoyl choline when fixation included 10 per cent formalin; *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis*, respectively; reactions in *A. proteus*, exceptionally weak. ×290.



Pretreatment with boiling water (FIGURE 103), iodine (FIGURE 104), or phenol completely eliminated the coarse cytoplasm deposition and lowered food vacuole and fine cytoplasm intensities, although levels of coloration remained. Deficiency media lacking substrate or both substrate and calcium resulted in the absence of coarse deposition, as with the monostearate ester but, unlike the monostearate ester, deficiency media lacking only calcium (FIGURE 105) or with calcium reduced (FIGURE 106) resulted also in absence of coarse deposition. In all of these deficiency media, food vacuole and fine cytoplasm intensities were lowered, although levels of coloration remained.

In the experiments with the monostearate ester, sparse loosely textured coarse deposition appeared with reduced calcium and even in the absence of calcium. The failure of coarse deposition with the present ester under these conditions may have been due to one or more of the following reasons:

(1) The simplest explanation derives from the difference in melting points between stearic and oleic acid. With the monostearate ester, even in the absence of precipitating cation, the liberated stearic acid, which is insoluble in water (Hodgman and Lange, 1930) and probably sufficiently insoluble in the incubation medium at 37° C., may have accumulated at or near loci responsible for the coarse deposition. Subsequent treatment with lead and with sulfide would then have transformed the fatty acid to lead sulfide, the indicator of sites of fatty acid deposition, even without intermediate calcium precipitation. Oleic acid, too, is insoluble in water; granting even similar insolubility in the incubation medium, the decisive difference between the stearic and oleic acids might well reside in the difference in melting point. The melting point of stearic acid is 69.2° C. (Hill and Kelley, 1943), well above the temperature of incubation, while the melting point of oleic acid is 14° C., well under the temperature of incubation. At the temperature of incubation, then, the liberated stearic acid might be expected to crystallize and to remain localized, while the liberated oleic acid might be expected not even to reach solid state.

(2) An additional factor may derive from differences in solubility between the two acids. Both are given as insoluble in water, it is true, but whereas stearic acid is soluble in alcohol to the extent of only 2.5 per cent and is very soluble in ether, oleic acid is soluble in alcohol and in ether in all proportions (Hodgman and Lange, 1930). This may indicate that insolubility in water or in incubation medium containing the mother substrate that is an ester of a complex alcohol, too, is relative, and that the oleic acid has, indeed, a degree of insolubility less than that of stearic acid. For microscopic cytochemical purposes, where fatty acid molecules needed to accumulate locally at 37° C. for many hours before the visibility threshold was crossed, such differences in solubility may have been decisive.

(3) There was also evidence of a leaching effect in the monooleate medium, in the sense that levels of nonspecific coloration were reduced as compared to similar nonspecific levels in the monostearate medium. Thus the levels, after incubation with a medium lacking both calcium and substrate, were higher than those after using a medium lacking calcium, but containing substrate, when monooleate was the substrate, but not when the monostearate was the

substrate; yet the experiments with both substrates were performed at the same time, under the same conditions, and were the same even as to mole concentrations of the esters. The heat and the phenol inactivation levels, too, were consistently lower after the oleate medium as compared with the stearate medium, pointing to a leaching effect on some basal level in the former. Monooleate and monostearate solutions of the same molar concentration were examined for surface activity. Compared with the monostearate solution, the monooleate solution foamed considerably more, and registered lower surface tension on the Du Nuoy tensiometer. While a leaching effect might be attributed to the slightly greater detergency of the oleate solution as compared to the stearate solution, another effect of greater surface activity might be to delay the precipitation of calcium oleate.

Among the differences between the reactions with the monostearate and monooleate esters were the character of the coarse deposition (brush-heaps with the monostearate and disks with the monooleate), the absence of any coarse deposition in the absence of cation with the monooleate (even in the presence of reduced cation), the possibly greater "leaching" effect of the oleate ester on some of the nonspecific levels of coloration, and the results with cobalt substitution for calcium followed by passage through lead and sulfide solutions. With the monostearate ester, cobalt substitution for calcium, followed by intermediate passage through lead, resulted in coarse deposition that was, however, smaller, less numerous, and more finely grained. The same treatment with the monooleate ester resulted in the complete absence of coarse deposition. Cobalt might be expected to inhibit enzyme activity toward the monooleate ester at least as much as toward the monostearate ester, if not more, for the monooleate ester is considered by some to be more specific with respect to "true" pancreatic lipase (Gomori, 1952*a*); the complete elimination of coarse deposition would be in accord.

The morphology of the coarse deposition appears to be rooted in the crystal pattern of the stearate and of the oleate under conditions obtaining. The monooleate of polyoxyethylene sorbitan is a liquid at room temperature but, on microscopic examination of the turbidity produced after several hours in the freezing compartment or overnight storage in the refrigerator, a turbidity that disappears with exceeding rapidity at room temperature, innumerable translucent disks with radial venation are observed. Under the microscope, these rapidly melted away, leaving spokes without a rim, so to speak, before disappearing completely. Under these conditions, then, the oleate needles aligned themselves radially to produce disk effects. The disks that represented coarse deposition after incubation in oleate medium accordingly appear to represent sites of activity of such nature as to result in calcium-oleate deposition in the above type of oleate pattern that is then reproduced in faithful replica as lead sulfide.

There appears to be no argument for the nonenzymatic origin of the coarse deposition. Their intracytoplasmic nature is demonstrated by their presence at various levels and by their occasional "streaming" arrangement in the cytoplasm.

The most noteworthy effects produced by diagnostic chemicals present in the incubation mixture were an outstanding inhibition by quinine and an occasional activation by taurocholate.

Chilomonads from culture or in food vacuoles were among organisms with lowest intensity levels and, although little affected by most types of treatment, they were slightly higher in intensity than after inactivation and deficiency media. Cirrous ciliates, *Colpudia*, and euglenoids were of low-level intensities, but in food vacuoles their intensities were often quite high. *Paramecia* and *Spirostoma* were infrequently observed after processing and were of low-level intensities not exceeding inactivation levels, hence negative; occasionally recognized in *Pelomyxa* food vacuoles, their intensities were considerably higher. Rotifers were of surprisingly low intensity and contained occasional spots of somewhat higher intensity under different conditions, but unfortunately these spots were not observed after normal (FIGURE 107) processing.

Aelosoma feeding on bottom debris was of a low-level body wall intensity similar to or lower than that with the monostearate ester and, generally, at inactivation levels or below, hence negative. The middle third of the annelid contains an expansion of the gut in which, by contrast, deposition was intense (FIGURE 108).

Since *Aelosoma* was observed to be a voracious bottom-debris feeder, it was of interest to compare the reactions obtained in the gut expansion with those of the coarse deposition in *P. illinoisensis* and of the bottom debris, all from the very same culture bowl (TABLE 9).

There appeared to be insufficient correlation between the effects of the above differentiating agents on the three types of coarse deposition, especially on the coarse type of deposition in *P. illinoisensis*. Refer also to additional remarks made in connection with the stearate ester, including observations on debris and ova reactions; they apply equally well here.

Myristoyl Choline

Microscopic enzyme chemistry, as it applies to this fifth of the five esters in the Gomori classification of carboxylic esterases, depends on the precipitation of cobalt myristate as the result of specific activity toward the soluble choline ester. Cation exchange is by-passed, the sites of activity being visualized as cobalt sulfide after direct anion exchange with sulfur. Experience with the monostearate and monooleate esters, with which avoidance of cation exchange with lead produced little if any specific deposition when cobalt was substituted for calcium—due perhaps to the notable inhibiting effect of cobalt on lipase—suggested that deposition after incubation with a medium containing myristoyl choline as substrate might be regarded all the more as the result of mediation of a different type of enzyme.

With the acetate of 2-hydroxy-3-naphthoic acid anilide, with the monostearate of polyoxyethylene sorbitan and with the monooleate of polyoxyethylene sorbitan, the method of fixation was that found best for beta-naphthyl acetate. These suitably timed combinations of primary fixation in chilled acetone followed by secondary fixation in chilled 10 per cent formalin were found to be

TABLE 9

	<i>Pelomyxa</i>	<i>Aeolosoma</i>	Debris
Normal	6.6 (medium)	7 to 8 (large)	7 to 8 (small)
Heat	0.0	—	2 to 3
Iodine	0.0	4	7
Phenol	—	3 to 4	6
No calcium-no substrate	0.0	3	3
Reduced calcium	0.0	3	8
No substrate	0.0	3	4 to 5
Cobalt to lead to lead sulfide	0.0	8	8 to 9 (fuzzy)
Arsenilate (2×10^{-4} M)			
During incubation	0.8*	8 to 9 (large)	8 to 9
Prior to and during incubation	1.6*	8	8 (small)
Arsenilate (10^{-3} M)			
Prior to incubation	1.7*	8 to 9 (large)	8 to 9
Prior to and during incubation	0.0	9 (large)	9 (small)
During incubation	1.3*	5 (large)	5 to 8 (small)
Eserine (10^{-6} M)			
Prior to incubation	3.3†	—	7 to 8
During incubation	2.3†	6 to 7 (large)	6 to 9 (small)
Prior to and during incubation	2.2†	8 (large)	5 to 8 (small)
Fluoride (2×10^{-3} M)			
Prior to incubation	4.2 (few)	8 to 9	4 to 6 (small)
Quinine (5×10^{-3} M)			
During incubation	0.0	8	6 (small)
Prior to and during incubation	0.0	8 (few scattered)	6
Quinine (2×10^{-3} M)			
Prior to incubation	0.0	5‡	5 to 7
During incubation	0.0	3	8
Prior to and during incubation	0.0	3	7 (jagged)
Taurocholate (2×10^{-3} M)			
During incubation	6.7 (few)	9 (few)	8 to 9
Prior to and during incubation	1.2 (very few, or absent)	9 (few)	9
Taurocholate (2×10^{-2} M)			
Prior to incubation	0.0	8 to 9	8 to 9 (few, large)
During incubation	0.0	3	9 to 10 (fuzzy)
Prior to and during incubation	0.0	3	6 to 7 (fuzzy)

* Very few and small, absent in most.

† Few or absent.

‡ Very few, large, or absent.

compatible with both large-scale processing and satisfactory intensities. With myristoyl choline, partly because of the greater lability attributed to cholinesterases (Augustinsson, 1950; Chessick, 1954) and partly because of the absence or paucity of deposition with the Tween substrates in the presence of cobalt, it was considered desirable to check further on the effects of fixation where cobalt is normally the precipitating cation and where direct sulfiding is normal to the method.

Reactions in the four species after fixation as with the other esters are shown in FIGURES 109 to 112. Cytoplasmic intensities in *P. illinoisensis* were on the low side. The reaction lag in *A. proteus*, extending even to the food vacuoles, is remarkably demonstrated. It is opportune to recall at this time that *A. proteus* reactions were remarkably weak with all esters except with beta-naph-

thyl acetate; with the latter, *A. proteus* reactions were stronger than in *A. dubia*. This observation was of decided importance for several reasons. A different route in carboxylic ester metabolism in *A. proteus* would be suggested. The strong reactions in *A. proteus* with beta-naphthyl acetate might be compensatory in nature. The duality (Gomori, 1952a) of the enzymes splitting the acetates of beta-naphthol and 2-hydroxy-3-naphthoic acid anilide would be supported by the inverse relationship between the two amoebae with respect to these esters. The weak reactions in *A. proteus* food vacuoles would suggest that at least part of the food vacuole intensities were contributed by the amoebae.

It was therefore considered desirable to check on the weakness of *A. proteus* toward myristoyl choline by comparing results obtained with a new culture from Dawson's laboratory. The effects of eserine sulfate, eserine salicylate (sodium salicylate controls), and prostigmine bromide were included in the study. The concentrations used were 10^{-5} M, this concentration having been used both prior to and during incubation with eserine (Chessick, 1954) and prostigmine (Gomori and Chessick, 1953). Acetone is said by some to abolish most or all specific cholinesterase activity at the myoneural junction except in the mouse (Chessick, 1954). Ten per cent formalin for 24 hours at 4°C. had been cited as preserving specific cholinesterase activity at the motor end plate even better than freezing and gelatin-embedding (Chessick, 1954), except at the lobster (abolished) and frog (reduced) myoneural junctions. Several methods of fixation were therefore tried with and without eserine to check the possibility that eserine-insensitive/eserine-sensitive enzyme ratios, if such there were, might be differentially affected, so that, in some cases, eserine-insensitive enzymes might no longer mask the expression of, and the effect of, eserine on the eserine-sensitive enzymes. Another measure was to use various combinations of threefold cobalt concentrations that had been found to produce negative results with the lipases when followed by the use of direct sulfiding, arsenilic acid that had been found to be outstandingly inhibitory in the case of the simple esterases, and of eserine, which is highly inhibitory for cholinesterase in most mammalian species.

Resistance to eserine with palmitoyl choline has been reported and attributed to possible decomposition of eserine with the obligatory prolongation of incubation with this ester (Gomori, 1952a). Accordingly, prolonged incubation for 60 hours was undertaken with myristoyl choline with and without changes of eserine. The effect of several periods of incubation, including 12, 24, and 36 hours, was tested with all of the variables mentioned above in order to determine whether or not some nonspecific enzyme was capable of masking the effect of the enzyme more specific for myristoyl choline, the more specific enzyme being expected to mediate earlier the deposition of the cobalt sulfide end product.

The results may be summarized as follows: (1) the weakness of *A. proteus* was confirmed at all incubation times regardless of whether *A. proteus* was cultured in the laboratory of Kopac or Dawson and regardless of the method of fixation; (2) acetone for 24 hours followed by 10 per cent formalin for 24 hours generally was far superior to acetone, alcohol, or alcohol used for 24

hours followed by formalin for 24 hours, and to formalin alone; (3) after 24 hours, normally incubated *A. proteus* from both cultures showed no clear-cut increase in intensities over deficiency media levels regardless of the method of fixation, while normally incubated *A. dubia* showed clear-cut net levels after fixation in acetone or acetone followed by formalin; (4) after 24 hours of incubation, *A. proteus* showed a negligible net level after acetone followed by formalin or formalin alone; *A. dubia* showed clear-cut net levels with all fixatives except alcohol; (5) after 36 hours of incubation, *A. proteus* showed small net levels in food vacuoles after acetone and after acetone followed by formalin, and in the cytoplasm after acetone followed by formalin. *A. dubia* showed clear-cut net levels, the intensities in the food vacuoles decreasing with fixation in this order: acetone followed by formalin, formalin (far inferior), acetone, alcohol followed by formalin, and alcohol (very little more than deficiency media levels). The intensities in the cytoplasm decreased with fixation in this order: acetone followed by formalin, acetone (far inferior), formalin, absolute alcohol followed by formalin, and alcohol (not more than deficiency media levels); (6) colorless crystal deposition was observed in *A. dubia*, but not in *A. proteus* after 36 hours of incubation in deficiency media containing myristoyl choline but lacking cobalt (this phenomenon will be discussed); (8) with optimum fixation, progressive increases in food vacuole and cytoplasm intensities were obtained with 12, 24, and 36 hours of incubation in both cultures of *A. proteus*. The same was true of the cytoplasm intensities in *A. dubia*; the increase after 36 hours was slight, but the food vacuole intensities reached maximum after 24 hours and were slightly decreased after 36 hours; (9) inhibition by 10^{-5} M eserine sulfate was far from total regardless of the method of fixation, time of incubation, species and origin of culture; (10) with incubation for 60 hours, there appeared to be no correlation between the inhibitory effect of eserine and the frequency with which the eserine was renewed, some of the highest intensities having been obtained with the greatest number of changes. Highest food vacuole intensities were now observed in *A. proteus*, higher even than in *A. dubia*, but the cytoplasm intensities were still much weaker. The mechanism of intensity regression with increased deposition has been discussed in connection with the stearate ester; (11) there appeared to be little difference in effectiveness between eserine sulfate, eserine salicylate, and prostigmine bromide; (12) a threefold cobalt concentration that resulted in negative or greatly reduced reactions with the lipase substrates when followed by direct sulfiding, resulted in little inhibition; (13) arsenilic acid resulted in depression of the intensities, especially with the longer periods of incubation where differences were more clear-cut; (14) a combination of threefold cobalt and arsenilic acid resulted in considerable depression compared with normal incubation, although an occasional food vacuole and area of cytoplasm were quite intense; (15) a combination of arsenilic acid and eserine resulted in little difference from eserine alone or less depression than eserine alone, due possibly to interaction between groups with opposite ionic charge; (16) a combination of threefold cobalt, arsenilic acid, and eserine resulted in intensities that might be interpreted in the same way; (17) omission of the activating cations, calcium, manganese, and magnesium, resulted in intensities somewhat lower in *A. dubia* and in stock *A. proteus*, but the intensi-

ties in the Dawson culture of *A. proteus* were greater without the activating cations; (18) prolonged incubation (60 hours) need not necessarily be feared when low enzyme activity requires it (*A. proteus*), for satisfactory intensities may result with little difference in localization and sharpness of outlines, at least in the grosser subdivisions of large cells.

In the complete experiment the concentration of formalin was reduced to 6 per cent. The period of incubation was 36 hours. Both measures were designed to improve the reaction intensities because of the added factor of pre-incubation treatment with various chemicals that necessitated greater exposures to aqueous solutions and to water for purposes of control. Postincubation washing was for 60 min. for better differentiation between specific and non-specific coloration attending the more complete removal of nonspecific cobalt. Cobalt acetate furnished the precipitating cation instead of cobalt chloride because of the decreased nonspecific cobalt binding. Three times the normal cobalt concentration was included as a variable because this was the concentration that resulted from equimolar substitution of cobalt for calcium in the Tween experiments in which this concentration was inhibitory. Three times the substrate concentration was included as a variable because of the reputed inhibitory effect of concentrations of this order on choline esterase (Gomori, 1952a). Another choline ester, lauroyl choline, was included as a variable in the present experiment, but will be referred to in a following section. Deficiency media lacking cobalt were of interest not only because information was furnished on one of the basal levels of coloration, but also because, with cation exchange for lead by-passed as is normal to the myristoyl choline method, colorless crystals had been detected in preliminary experiments.

In this experiment a coarse cytoplasmic concomitant was present in *A. dubia* and in *P. carolinensis* and infrequent or absent in *P. illinoisensis* and in *A. proteus* (FIGURES 113 to 115). Nuclear staining was never observed. The species in decreasing order of net food vacuole intensities were: *P. carolinensis*, *A. dubia*, *P. illinoisensis*, and *A. proteus*. The species in decreasing order of net intensities for the over-all cytoplasm were: *P. carolinensis*, *A. dubia*, *P. illinoisensis*, and *A. proteus* (difficulty was experienced in separating fine from coarse cytoplasm intensities).

Pretreatment with boiling water (FIGURE 116) and with phenol completely eliminated the coarse concomitant from the cytoplasmic deposition in the

FIGURES 113 to 115. Reactions with myristoyl choline when fixation included 6 per cent formalin; reactions in *A. dubia*, *P. carolinensis*, and *P. illinoisensis*, respectively. $\times 290$. FIGURE 116. *P. carolinensis*. Pretreatment with boiling water prior to incubation with myristoyl choline. $\times 290$. FIGURE 117. *P. carolinensis*. Deficiency media lacking substrate. $\times 290$. FIGURE 118. *P. carolinensis*. Deficiency media lacking both cobalt and substrate. $\times 290$. FIGURES 119 to 123. Deficiency media lacking cobalt, but containing myristoyl choline; colorless crystals appear when high activity is indicated under normal conditions in incubation, but not when activity is normally negligible. $\times 290$. (119) *A. dubia* (colorless crystal observed). (120) *A. proteus* (no colorless crystals observed). (121) *Paramedium* (colorless crystals observed). (122) *Spirostomum* (no colorless crystals observed). (123) Debris (colorless crystals observed). FIGURES 124 and 125. Threefold cobalt, a concentration productive of negligible deposition with the lipase esters; *A. dubia* and *P. carolinensis*, respectively. $\times 290$. FIGURES 126 and 127. Eserine inhibition; *A. dubia* and *P. carolinensis*, respectively. $\times 290$.

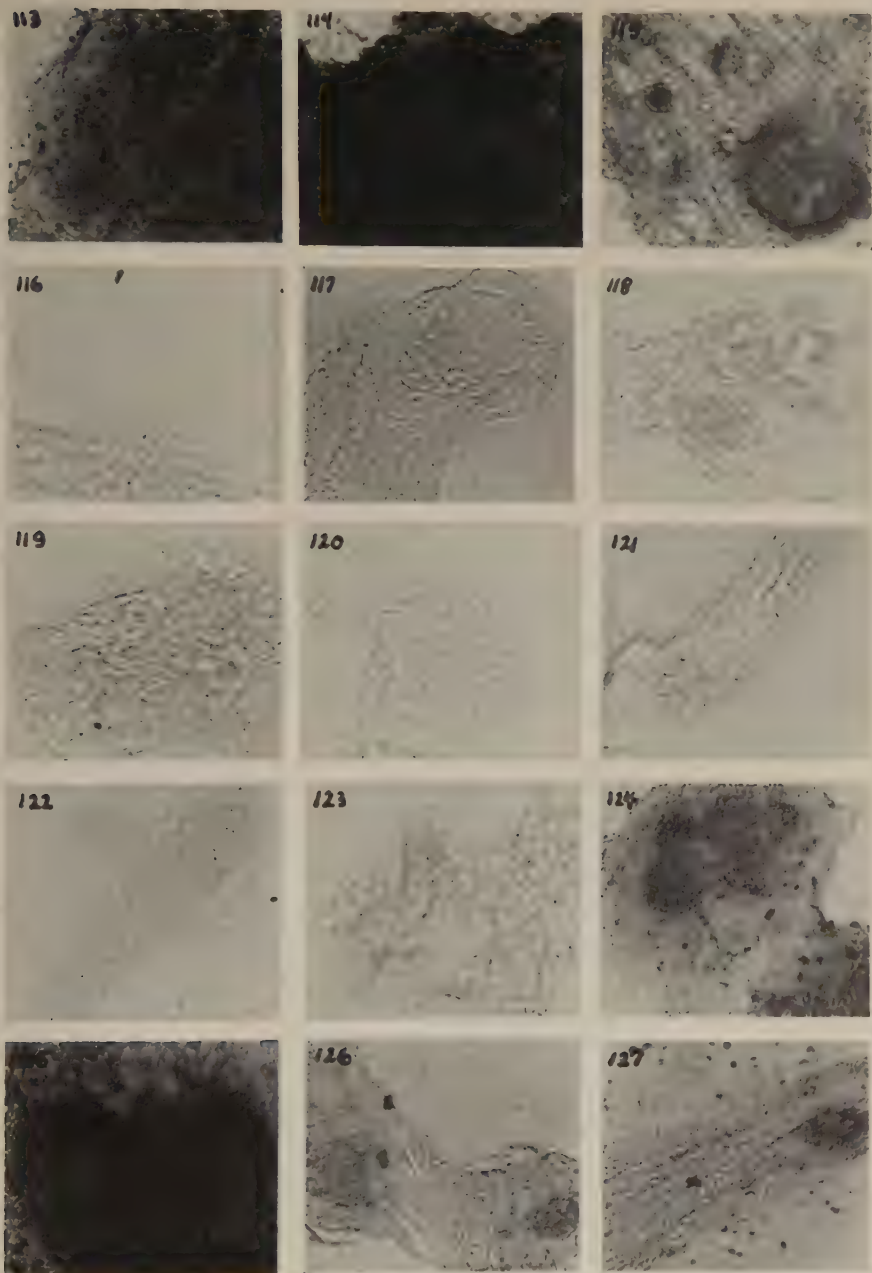


TABLE 10

	Solubility in water	Solubility in alcohol	Solubility in ether	Melting point
Myristic acid	Insoluble	Very slight	Very slight	53.8° C.
Oleic acid	Insoluble	Soluble in all proportions	Soluble in all proportions	14.0° C.
Stearic acid	Insoluble	2.5%	High	69.3° C.

species in which the coarse deposition occurred (*A. dubia* and *P. carolinensis*) and this treatment lowered food vacuole and other cytoplasm intensities, although levels of coloration remained. Unlike the coarse deposition with Tween 60 (monostearate) and Tween 80 (monooleate), the coarse concomitant was not completely eliminated by pretreatment with iodine. Deficiency media lacking substrate (FIGURE 117) or both cobalt and substrate (FIGURE 118) resulted in the absence of the coarse concomitant and in a lowering of food vacuole and cytoplasmic intensities, although levels of coloration remained. The levels of coloration in the food vacuoles and in the cytoplasm were lower after heat inactivation than after phenol inactivation in all species; this was unlike the relative values with the monostearate and monooleate esters, with which the levels were very similar. The efficacy of the postincubation wash was indicated by the similarity in levels after media containing neither cobalt nor substrate and media containing cobalt, but no substrate.

With media containing substrate but no precipitating cobaltous cation, there appeared in the amoebae generally (FIGURE 119) but not in *A. proteus* (FIGURE 120), in *Paramecium* (FIGURE 121) but not in *Spirostomum* (FIGURE 122), and in the debris from all cultures (FIGURE 123), a remarkable type of deposition, quite ample yet colorless, and wholly predictable if the previously given interpretation of the nature of the colored deposits with Tween 60 and the absence of deposits of any sort with Tween 80 under similar conditions was correct.

Comparison between salient physical properties of the three fatty acids (Hodgman and Lange, 1930) suggests that deposition of myristic acid is to be expected in the absence of cobaltous ions (TABLE 10). Also expected is the absence of color, since cation exchange for lead is not normal to the method.

A threefold concentration of cobaltous acetate—a concentration equal to equimolar substitution of cobalt for calcium in the tests for lipase where direct sulfiding resulted in complete elimination of the net intensities in the food vacuoles and in the cytoplasm—although productive of somewhat finer deposition, resulted in considerable intensities (FIGURES 124 and 125), thus suggesting that the enzymes for the Tween substrates, on the one hand, and the myristoyl choline substrate on the other, were different.

A threefold increase in substrate concentration, an increase that resulted in a concentration of 1.2×10^{-3} M, an inhibitory concentration for activity against myristoyl choline (Gomori, 1948, 1952a), resulted in 30 to 100 per cent decreases in net intensities in the food vacuoles and 40 to 100 per cent decreases in net intensities in the over-all cytoplasm deposition with both diminution and loss of coarseness in all species.

TABLE 11

	Food vacuoles				Cytoplasm, over-all				Coarseness			
	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>	<i>A.d.</i>	<i>A.p.</i>	<i>P.c.</i>	<i>P.i.</i>
Eserine prior to and during incubation												
2×10^{-6} M	-62	(c)	-57	-100	-88	(c)	-57	-100	None	(a)	None	(b)
10^{-4} M	-98	-100	(c)	-100	-100	-100	(c)	-100	None	(a)	(c)	(b)
Eserine during incubation												
2×10^{-6} M	-59	-100	-71	-100	-76	-100	-79	-100	None	(a)	None	(b)
10^{-4} M	-80	-67	-43	-100	-91	-94	-36	-100	None	(a)	Finer	(b)
Eserine prior to incubation												
2×10^{-6} M	-25	-33	-31	-60	-33	-83	-36	-74	Less	(a)	Scattered	(b)
10^{-4} M	-7	-61	-43	-73	0	-94	-15	-100	Rare	(a)	Scattered	(b)

(a) No coarse concomitant observed in *A. proteus*.(b) The coarse concomitant in *P. illinoisensis* was sparse or absent.

(c) All animals were lost from cover slips.

When fixation had included 10 per cent formalin, coarse deposition tended to be absent and eserine resistance was noted. In the complete experiment, after 6 per cent formalin, coarse deposition was present, especially in *A. dubia* and *P. carolinensis*, and considerable inhibition was obtained with eserine (FIGURES 126 and 127). The percentages of change from normal, of the numerical averages of the food vacuole intensities, and of the over-all cytoplasm intensities in the 4 species of amoebae are shown in TABLE 11.

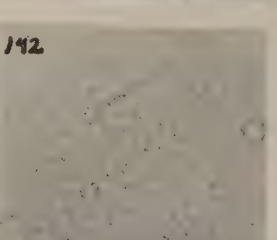
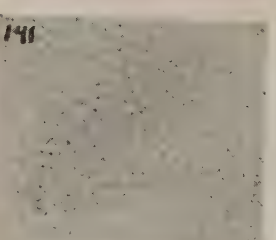
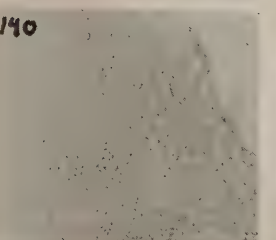
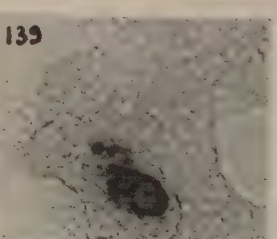
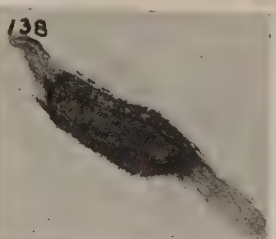
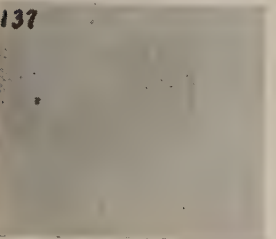
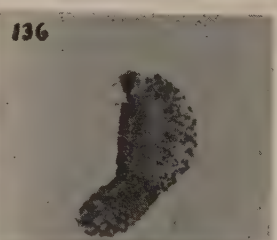
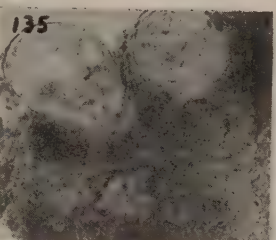
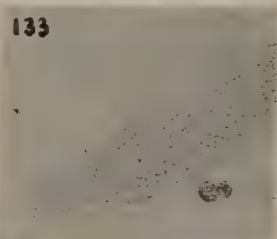
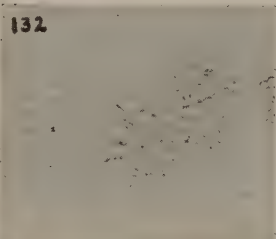
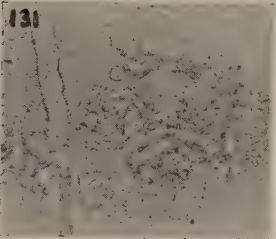
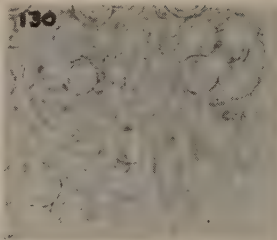
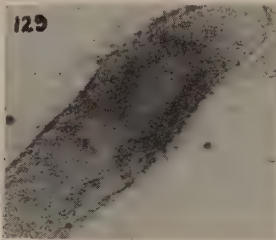
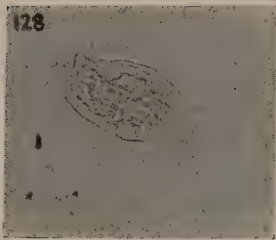
All of the other diagnostic agents were also highly inhibitory, although none was used in as low a concentration as eserine, and none in concentrations of 10^{-2} to 10^{-4} M were as inhibitory as eserine in 10^{-4} M concentration.

Quinine, which was frequently activating with the acetate esters, was never activating with the choline ester when present in the incubation medium. Taurocholate, which was occasionally activating with the stearate and oleate esters, was never activating with the choline ester when present in the medium.

Cobalt in a concentration highly inhibitory with the stearate and oleate esters did not prevent the accumulation of considerable specific deposition with the choline ester.

Chilomonads from culture or in food vacuoles were at general inactivation levels. Cirrous ciliates (FIGURE 128), *Colpidia*, and euglenoids were generally of low intensity with small net values over phenol inactivation levels; in amoeba food vacuoles, their intensities were greater. Paramecia were observed most frequently in *P. carolinensis* and *P. illinoisensis* cultures and were of intensities considerably above general inactivation values (FIGURE 129). The intensities of the paramecia in culture were below the intensities observed in food vacuoles in *P. carolinensis*, but were of the order of food vacuole intensities observed in *P. illinoisensis*.

Of considerable interest are the colorless crystal deposits in the cytoplasm of *A. dubia* (FIGURE 119), but not in *A. proteus* (FIGURE 120), despite their



presence in debris from *A. proteus* culture (FIGURE 130) and in debris from all other cultures (FIGURES 123 and 131) when cobalt was absent but substrate present. They were observed in paramecium (FIGURES 121, 132) under the same conditions but not in *Spirostomum* (FIGURE 122). *Spirostoma*, observed only in *P. carolinensis* cultures, were considerably lighter (FIGURE 133).

The normal intensity and the colorless crystal relationships between *A. dubia* and *A. proteus* were similar, then, to those between *Paramecium* and *Spirostomum*; colorless crystals in the absence of cobalt constituted, then, an unusual sign of high enzyme activity.

Rotifers were of surprisingly low intensity, but occasionally contained spots of somewhat higher intensity (FIGURE 134) that tended to correlate with debris intensities. Observed occasionally in food vacuoles and in debris, the rotifers were conspicuous by their pale contrast with the surrounding deposition (FIGURE 135). An unusual metazoan was present in *P. carolinensis* culture; striking intensities with myristoyl choline were displayed (FIGURES 136 and 137). *Aeolosoma* feeding on bottom debris and present only in the *P. carolinensis* culture demonstrated body intensities (FIGURE 138) somewhat above even iodine-inactivation levels. The middle third of this animal contains an expansion of the gut in which were obtained reactions of striking intensity. Unfortunately, it was not determined at the time whether the small node of nervous tissue in the anterior part of the animal was stained. Information on correlative *Pelomyxa*, *Aeolosoma*, and debris reactions tended to be fragmentary, but there appeared to be better correlation between debris reactions and reactions in *Aeolosoma* than with the stearate and oleate esters. Correlation between debris reactions and reactions in *P. carolinensis* cytoplasm were not as good. There was practically no correlation between debris reactions and coarse cytoplasm reactions in the other amoebae. Debris reactions could not be made to account for the food vacuole reactions, notable contradictions being frequent if attempts were made to relate the appearance and actual intensities of, or the effects of chemicals on, the debris and food vacuole intensities.

Lauroyl Choline

The cholinesterase demonstrated by the myristoyl choline technique is generally described as more active with lauroyl choline and less active with palmitoyl choline than with myristoyl choline. Thus, the recommended incuba-

←
FIGURE 128. Cirrous ciliate; negligible reaction with myristoyl choline. $\times 290$. FIGURE 129. *Paramecium*, considerable activity with myristoyl choline. $\times 290$. FIGURES 130 and 131. Debris from *A. proteus* and *Pelomyxa* cultures showing crystals in the absence of cobalt; *A. proteus* from the same culture develops no colorless crystals under the same conditions. $\times 290$. FIGURE 132. As in FIGURE 121. $\times 290$. FIGURE 133. *Spirostomum* showing negative reactions with myristoyl choline under normal conditions of incubation; absence of colorless crystals in *Spirostomum* has been noted in FIGURE 122. $\times 290$. FIGURE 134. Rotifer with little activity towards myristoyl choline except for some inclusions (?). $\times 290$. FIGURE 135. Rotifers showing negligible reactions, although embedded in debris of high intensity. $\times 290$. FIGURE 136. An unusual organism found only in *P. carolinensis* culture showing high activity with myristoyl choline. $\times 290$. FIGURE 137. Same as above under the influence of arsenilate (eserine was also highly inhibitory). $\times 290$. FIGURE 138. *Aeolosoma* reactions with myristoyl choline. $\times 70$. FIGURES 139 to 142. Reactions of the four species with lauroyl choline; *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis*. $\times 290$.

tion times at 37° C. are 6 to 12 hours with lauroyl choline, 12 to 24 hours with myristoyl choline, and 24 to 48 with palmitoyl choline (Gomori, 1952a). With the naphtholic substrates, negative or faint reactions were obtained with beta-naphthyl laurate. The same weakness was observed with the laurate ester of polyoxyethylene sorbitan (Tween 20) in preliminary experiments with the Tween technique. In the complete myristoyl choline experiment, incubation was carried out at and for the same time in lauroyl choline medium. This incubation period should have been more than sufficient for lauroyl choline, but the reaction intensities were extremely weak (FIGURES 139 to 142).

(1) There was complete absence of the coarse concomitant in the species in which it appeared with myristoyl choline; (2) the net intensities in the food vacuoles as compared with myristoyl choline were reduced 64, 100, 93, and 100 per cent in *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis*, respectively; the net intensities in the over-all cytoplasm, as compared with myristoyl choline, were reduced 88, 100, 93, and 100 per cent in *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis*, respectively; (3) *A. dubia* appeared to be the most active with this choline ester; (4) the intensities were reduced in other organisms that had shown net intensities with myristoyl choline; (5) the intensities in the debris were likewise reduced.

Discussion

Processing. Rapid fixation was necessary to preserve form. Formalin was deficient, but alcohol and acetone were more satisfactory in this respect. Alcohol fixation resulted in negative or feeble enzyme reactions. A number of enzymes are soluble in alcohol or in acetone (Nachlas *et al.*, 1957). Alcohol may denature proteins by an outward turning of the chains and resulting rupture of linkages (Pearse, 1953). A new group of protein lipids soluble in organic solvents and insoluble in water has been reported (Folch and Lees, 1950). Whether the unfavorable process is due to leaching into the alcohol, denaturation, steric disruption, or inaccessibility attending protein precipitation, it was halted to some extent when formalin was present in the alcohol. The resulting intensities were high, but patchy and gradient.

Acetone fixation resulted in improved enzyme reactions, but these were still weak. Acetone containing formalin produced weak, patchy reactions that might be attributed to an insoluble residue formed in the mixture and left behind in drying. The weak reactions after acetone fixation may be due to extraction of enzyme in aqueous media that followed (Doyle and Liebelt, 1954). For the same reason, probably, animals dried without fixation not only exhibited poor morphology but also negative to weak reactions.

Formalin as part of the fixation process had shown promise. The interactions between formaldehyde and proteins are numerous and complex (French and Edsall, 1945); many are known, but most are inferred from experiments with amino acids. Addition and condensation reactions take place with amino, imino, and amide groups to form hydroxymethyl compounds that may combine with each other to form simple methylene bridges, intramolecular cyclic compounds, and intermolecular aggregates. These, in turn, may combine with

additional formaldehyde molecules to form polyoxymethylene chains or bridges. The situation is still more complicated with polyfunctional amino acids and probably vastly more so with proteins. Addition and condensation compounds may form with hydroxyl and carboxyl groups, and even with peptide links. Guanidino groups are erratically altered in a way yet unknown. Thio analogues may be formed with sulfhydryl groups. Formaldehyde, however, may act as a reductant for disulfide linkages; the carboxylic esterases are dependent for their activity on sulfhydryl groups.

While formalin probably destroys some esterase activity, the remainder appears to be nonextractable (Doyle and Liebelt, 1954). With various choline esters, clear and precise pictures have been obtained after neutral formalin at room temperature, although there were species and tissue differences (Chessick, 1954).

A combined procedure suggested itself—that of primary fixation in acetone, followed by drying on cover slips followed by secondary fixation in formalin. Rehydration of dried animals with a drop of lightly hydrated acetone followed by secondary drying resulted in better affixture.

Following double fixation the reactions were intense, fine grained, and uniformly distributed. Ten per cent formalin was somewhat superior to 5 per cent. With the myristoyl choline procedure, 6 per cent formalin resulted in more coarse deposition and in surface encrustation particularly noticeable in *A. dubia*. The deposition was more eserine-sensitive than after fixation in 10 per cent formalin.

Processing large numbers of animals demanded cover slips as carriers. Processing of loose animals resulted in large losses, difficulties due to interface trapping in passing from solutions of low to high density, and an inability to wash thoroughly, to adhere to time schedules, or to process several different types of organisms at the same time. Food organisms and debris could be processed simultaneously.

The method that evolved avoided the adverse effects of affixitives that may oppose permeation by even simple and highly diffusible substrates (Zacks, 1953), avoided embedding and embedding temperatures (Gomori, 1952a), and avoided collodion, which was found to peel and is said to oppose permeation by unsaturated fatty acid esters (Gomori, 1952a) and to stain with diazonium salts (Pearse, 1953).

Rating of reaction intensities. Reaction intensities of this sort, generally, are roughly and subjectively graded. This may account for discrepancies in the literature. In all complete experiments, a ten-grade comparator wheel greatly facilitated subsequent analysis, including detection of minimal intensities and information on sources of several nonspecific levels of coloration inherent in the Gomori technique.

Difficulties in quantitation are inherent in the histochemical method and, as pointed out by Gomori, (1955) "one can only judge visually and in arbitrary units; differences in intensity of the order of plus or minus 30 per cent may escape the eye altogether."

Sources of nonspecific coloration: deficiency media and inactivation procedures. A principal potential source of nonspecific coloration with the Gomori technique resides in the high level of nonspecific endogenous cation. A positive test for magnesium has been detected in the center of the refractile body with a faint positive test at the periphery; considerable calcium has been detected in the refractile body and in the cytoplasm (Heller and Kopac, 1956).

With incubation by-passed, very intense reactions were obtained for endogenous cation capable of exchanging with lead and of being visualized as lead sulfide in the cytoplasm and in the refractile bodies; in the latter, the concentrations were either central or peripheral or both. The round bodies of ingested chilomonads and euglenoids were relatively noncolored. These round bodies differed also in that they were much more highly birefringent and tended to retain their morphology better after demineralization or prolonged exposure to water. The refractile bodies tended to become invisible after demineralization, as if structural integrity were calcium-dependent.

These endogenous sources of cation were largely eliminated by treatment with standard citrate buffer or one thousandth molar disodium Versenate in distilled water. Maximum reduction depended, however, on a suitable amount of washing following solvation and chelation. The greater complexing affinity of Versene for lead than for calcium and the ability of sulfide ion to liberate lead even from the chelate (Bersworth, 1952) are in accord.

With the Tween technique itself, sources of nonspecific coloration could be attributed to calcium binding in the medium and to subsequent lead binding. These sources were reduced by postincubation and postlead washing to levels obtained after media lacking cation. The amount of washing necessary was compatible with satisfactory reactions.

Comparable experiments disclosed that nonspecific binding of cobalt in substitution for calcium in a medium containing Tween was greater than nonspecific calcium binding. In media containing myristoyl choline, cobalt chloride was bound more strongly than cobalt acetate, confirming Gomori's preference for the acetate (Gomori, 1952a).

The low level of coloration remaining after media lacking both cation and substrate, even when subsequent cation treatment was not involved, may have been due to coloration in the living animal itself.

An understanding of sources of nonspecific coloration that resembled specific coloration in hue in the Gomori technique was deemed essential to the assignment of basal levels for the purpose of transformation of gross intensities to net intensities.

With the Seligman technique, demineralization is not essential, as the final reaction products are not metallic. Gross levels were at the same time net levels, especially with the diazonium salt employed, for nonspecific coloration was faint and of a hue complementary to the brilliant purplish specific shades.

Treatment for 15 min. with boiling water or 5 per cent phenol prior to incubation completely, or almost completely, abolished specific hues in the food vacuole and cytoplasm with the Seligman technique. The nonspecific coloration was altogether like that in noninactivated animals incubated in media lacking substrate. Pretreatment with Lugol's iodine (strong) resulted in re-

sidual coloration ranging from barely minimal in the amoebae to two thirds of normal in the pelomyxae in terms of comparator wheel units.

With the monostearate and monooleate esters of polyoxyethylene sorbitan and with myristoyl choline, boiling water or phenol completely eliminated the coarse deposition in those species in which it occurred; iodine eliminated the coarse deposition with the monostearate and monooleate esters, but did not completely eliminate it with the choline ester, indicating perhaps some difference in activity between the enzymes.

Food vacuole and fine cytoplasm deposition were greatly reduced by heat and phenol pretreatment with all the long-chain fatty acid substrates in all species. With the stearate ester, heat inactivation levels were somewhat higher than were phenol inactivation levels due, possibly, to alteration in cation binding rather than residual activity (with the naphtholic substrates they were the phenol, rather than the heat-inactivation levels, which produced slight residual intensity). With the monooleate ester, heat and phenol inactivation levels were quite similar, due possibly to the demonstrated greater surface activity of this Tween.

With iodine pretreatment, food vacuole and cytoplasmic levels were higher than with heat or phenol pretreatment. The difference was probably in good part true residual activity for, with the naphtholic substrates, these levels were also the highest of the inactivation levels. Little or no part of the staining was due to the iodine, since sodium thiosulfate failed to reduce the coloration, nor was iodine staining noticeable with the Seligman technique.

Deficiency media lacking both substrate and cation, or substrate alone, resulted in the absence of coarse deposition with all substrates, and greatly reduced the levels of coloration. A low degree of nonspecific calcium binding in the Tween 60 medium was suggested by comparing results with these types of deficiency media. After Tween 80, this difference was negligible, due, perhaps, to greater inhibition of nonspecific calcium binding by the greater surface activity. A high degree of lead binding was suggested by comparison between levels of coloration after media containing neither cation nor substrate and followed by passage through lead (as in the Tween technique) with levels after media containing neither cation nor substrate followed by direct sulfiding (as in the myristoyl choline technique). Cobalt binding appeared to be of greater order than calcium binding, judging from the higher coloration level when cobalt was substituted for calcium in substrate-deficient media and cation exchange with lead included in the process.

Sources of specific deposition in deficiency media. In media containing monostearate at reduced calcium levels, the coarse deposition was lighter, sparser, and more loosely textured. Cytoplasmic intensities were greatly reduced, and food vacuole intensities were reduced to the partial levels after iodine pretreatment. In monooleate medium containing reduced calcium levels, both the fine cytoplasmic and food vacuole intensities were greatly reduced and the coarse deposition completely eliminated.

In appearance, the coarse deposition resembled the crystal structure of the mother substrate. The stearate at room temperature is a solid in equilibrium

with a liquid phase, and examination disclosed birefringent brush-heaps. The oleate at room temperature is a liquid but, after overnight refrigeration or freezing for one hour, rapidly melting disks were observed. The failure of coarse deposition with the oleate may have been due in part to a lesser degree of freedom in crystallizing (more two-dimensional), a delaying effect of the more surface-active oleate on precipitation, and the inability of the primary reaction product to contribute by remaining localized, as do stearic and myristic acid (even in the absence of calcium). A consideration of the method and a comparison between salient physical properties of the fatty acids (Hodgman and Lange, 1930) provide the explanation (TABLE 10).

Thus, even in the absence of cation, the coarse deposition with the stearate, the absence of coarse deposition with the oleate, and the colorless crystals with myristoyl choline reflected the degree to which the primary reaction product remained localized without help from precipitating cation. With the stearate, the stearic acid crystal itself was reproduced as lead sulfide while, with the oleate, no oleic acid crystallization was to be expected. With myristoyl choline the myristic acid crystals were not replaced by cation; hence, they were incapable of being darkened by sulfide.

The colorless crystals of myristoyl choline in the absence of cobalt were regarded as furnishing an unusual type of evidence in favor of enzyme mediation. Thus, *Paramecium*, *P. carolinensis*, *P. illinoisensis*, *A. dubia*, and debris from all cultures showed considerable net intensities in the normal myristoyl choline technique, while *Spirostomum* and *A. proteus* were weak or negative. In the absence of cobalt, colorless crystals appeared in *Paramecium*, in *A. dubia*, and in the debris (the pelomyxae were lost from the cover slips, but it is predicted that crystal deposition would be found), but not in *Spirostomum* and *A. proteus*.

The basal level for transforming gross to net intensities. The difficulty with the Gomori technique was the persistence of several nonspecific levels of coloration. The most logical basal level would appear to be one integrating as many as possible of these nonspecific levels. This would be expected to result from incubation in normal medium followed by normal processing, provided enzyme activity were nil. Closest to meeting this was the heat- or phenol-inactivation level.

Net deposition in the amoebae. Net levels were observed with all of the substrates involved in the Gomori microscopic enzyme cytochemical classification; in the food vacuoles and in the cytoplasm; but not in the nuclei which, when observed, appeared as light hyaline ovals or spheres.

With the naphtholic substrates, specific hues conformed to those described in the histochemical literature and were never obtained in the absence of substrate. Nonspecific hues increased in intensity with increase in temperature, pH, and buffer concentration, but did not interfere under the conditions employed and with the diazonium salt used.

Nonspecific coupling between diazonium salts and reactive protein groups, that is, the tyrosine phenolic, the tryptophan indol and the histidine iminazole, usually results in pale yellow coloration, although with some diazonium salts

and their break-down products, coloration is more intense (Pearse, 1953). With the diazonium salt employed, nonspecific coloration was of the paler variety. Nevertheless, if the diazonium salt were to attach itself to a site unrelated to enzyme activity and in such a manner as to leave reactive the group that couples with the product of enzyme activity, the possibility for false localization exists, especially since the naphthol appeared to be quite diffusible. Incubation in a medium containing the diazonium salt or substrate, each in the absence of the other, followed immediately by incubation in the complementary media—medium lacking substrate or lacking diazonium salt, respectively—resulted in complete absence of specific coloration. The amount of liberated naphthol retained was insufficient for detectable coloration when incubation was followed immediately by the diazonium salt. Conversely, the bound diazonium salt could not couple with the naphthol being liberated. There appeared, then, to be little danger of false localization on this score. The result suggested, in addition, that a very great turnover, coupled with simultaneous precipitation, was necessary before the threshold of microscopic visibility of the specific coloration could be crossed.

For cells of the size employed and for their gross subdivisions, localization with the naphtholic substrates appeared to be adequate with the method used, judging from sharpness of outlines of recognizable morphologic entities, separation between darkly stained food vacuoles and light areas of cytoplasm, separation between lightly stained food vacuoles and dark areas of cytoplasm, the presence of dark and light food vacuoles at pseudopodial tips separated from the main body by narrow protoplasmic bridges, and the sharp demarcation lines between contiguous or overlapping pseudopodia of different intensities.

These empirical criteria of adequate localization in the larger subdivisions of the protoplasm applied almost as well to the long-chain fatty acid substrates. The only difference appeared to be one of occasional physical encroachment by crystals onto morphologic demarcation lines.

The reactions with the corresponding laurates of the three classes of substrates were unsatisfactory. It is doubtful whether any specific deposition resulted from the use of beta-naphthyl laurate. In the hope that a competing substrate technique might display some surpassing sensitivity, beta-naphthyl acetate was used in concentrations that gave rise to barely perceptible specific coloration. The presence of normal concentration of beta-naphthyl laurate did not, with certainty, extinguish or reduce these levels. Lauroyl choline used as in the myristoyl choline technique likewise produced weak reactions, although more rapid splitting is generally observed in other species (Gomori, 1952a). Preliminary experiments with Tween 20, the laurate ester of polyoxyethylene sorbitan, likewise produced weak reactions. The low intensities resulting from these esters, which are of a chain length intermediate between that of esters productive of strong intensities, suggested a separation between the enzymes acting on the acetate and on the long-chain fatty acid esters.

Net intensities with all five major substrates were observed in the food vacuoles and cytoplasm of all species. The variability was sometimes considerable even between individuals from the same culture, but the over-all results in a

given species were similar, whether clone or stock and whether from the laboratories of Kopac, Dawson, or Daniels. Amoebae judged to be moribund tended not to be colored or to be greatly reduced in intensity.

With the Gomori technique, the intensities tended to pass through a maximum with time and then to decline. In *A. proteus*, with weaker initial intensities, the maximum was reached much later, generally at a time when the reactions in the other species were declining. The need for graded periods of incubation in the enzyme cytochemical technique was apparent. The decline was most dramatic in the food vacuoles that, initially, were the most intense. The phenomenon first appeared as a central pallor; with the monooleate, which was productive of lower intensities in general, the maxima appeared later.

The most likely explanation was viewed in the light of a reversal effect, similar to photographic "photo reversal," due to a deposition too compact for efficient cation and anion exchange. The remedy lies, perhaps, in the use of polarization microscopy, phase contrast, or some more efficient condition of exchange. A tenfold increase in calcium concentration and normal incubation tended to simulate prolonged incubation.

Types of deposition: the nature of the coarse deposition. Deposition was relatively fine with beta-naphthyl acetate and finer still with naphthol AS acetate. In *Pelomyxa* there was some coarsening. In tiny amoeboid forms of the *Vahlkampffia* and *Amoeba dofleini* type, deposition was so fine as to be unresolved at 580X. Cytoplasmic deposition with the stearate tended to be inseparably fine and coarse in *A. dubia*; fine with discrete, sparse, coarse sprinkling in *A. proteus*; and fine to heavily coarse, with many intermediate grades, in *P. carolinensis* and *P. illinoisensis*.

With the oleate, coarse deposition was as intense as with the stearate, but the fine deposition tended to be of lower net intensity, and the food vacuoles tended not to be as compactly opaque as with the stearate.

The coarse deposition may represent some combination of factors such as loci of more specific activity or concentrations of loci of activity, whether present in life or dislocated, aggregated, or trapped in cytoplasmic loculi. With certain types of preparation for incubation, such as prolonged washing, deposition was coarse even with beta-naphthyl acetate; with acetone fixation alone, the deposition with beta-naphthyl acetate was often coarse. Whether this was due to differential destruction or dislocation resulting in more isolated loci of activity (such disperse centers favor larger crystal growth) or whether it was due to dislocation and aggregation cannot be said at present. Certain types of chemical treatment during incubation that greatly diminished over-all intensities resulted in "vacuole aggregates," which appear to be clusters of darker granules appearing to lie in tiny vacuoles or cytoplasmic loculi; this may have been due to differential suppression, resulting again in disperse centers, or to activation of such disperse foci.

The initial crystal lattice may conceivably be formed at a given site as the result of heightened activity at that site. Assuming negligible diffusibility of the liberated products or the secondary products, increase in crystal size might be related to physiological activity; localization, not fine detail, could be assured.

Assuming a degree of diffusibility, it is conceivable that crystal-forming "nuclei" could be formed fortuitously as a result of chance collision between molecules—the more diffusible, the farther from the site of activity. Both the crystal "nuclei" at sites of activity and at fortuitous sites could then grow as a result of molecular trapping by the lattice, although these molecules may have originated elsewhere.

Coarse deposition with some surface encrustation was present and eserine sensitivity was enhanced in *A. dubia* incubated with myristoyl choline when the formalin concentration was lowered.

Measures expected to decrease diffusion of the reaction products reduced the coarse deposition, as has been noted with the tenfold calcium concentration; conversely, lowered calcium concentrations increased crystal size. In the absence of cobalt, the colorless crystals with myristoyl choline were exceptionally large and appeared to grow from one or few points, as has been noted with indigoid deposits enzymatically liberated from indoxyl acetate by fat cells (Barnett and Seligman, 1951).

The coarse deposition in the amoebae did not appear to be related to the coarse deposition in the debris, which was also enzymatically mediated, but apparently by several enzymes with different *pH* optima, so that a plateau tended to be maintained throughout the *pH* range studied; however, no such plateaus appeared in the cytoplasm. Taurocholate, moreover, appeared to be converted by the debris to a product precipitating with cation, as shown by the presence of coarse deposition in the debris in the absence of substrate. This type of deposition was absent in the cytoplasm, but occasionally appeared in the food vacuoles between the food organisms and the vacuole wall, although in a more restricted *pH* range, thus suggesting that fewer species of bacteria survived the gastric process. Penicillin, too, failed to alter the amount and distribution of the coarse deposition in the cytoplasm.

A metabolic pathway from taurocholate to calcium-precipitable sulfur compounds by way of taurine and cysteic acid (Umbreit, 1954) may find support in the observation that injected cysteic acid fails to give rise to urinary sulfate, whereas ingested cysteic acid does give rise to urinary sulfate, presumably through bacterial action (Fromageot, 1952).

The magnitude of the net intensities. In *A. proteus*, the intensities were high with beta-naphthyl acetate—higher than in *A. dubia*—but the intensity with the naphthoic acid anilide ester was negligible, much lower than in *A. dubia*. The intensities in *A. proteus* were considerably lower also with the stearate and oleate esters and with myristoyl choline, thus suggesting that the exceptional intensities with beta-naphthyl acetate might be compensatory in nature. In *A. dubia*, the cytoplasmic intensities were satisfactory with all of the substrates. In the *Pelomyxae*, the deposition tended to be most intense. In all the species, the intensities with the oleate ester tended to be lowest.

The inverse relationship between *A. dubia* and *A. proteus* with respect to the two acetates would appear to support Gomori's idea that two different esterases are involved (Gomori, 1952*a, b*).

A generic classification of amoebae on the basis of certain enzyme activities (Andresen and Holter, 1949), would either appear to be unwarranted, or else

A. proteus and *A. dubia* should be placed in separate genera. *A. proteus* would appear to be less closely related to the *Pelomyxæ* than *A. dubia*.

The biochemical classification. Carboxylic esterases are enzymes hydrolyzing carboxylic acid esters of alcohols and phenols. The biochemical classification is controversial, but depends largely on substrate preference and the effects of certain chemicals considered to be diagnostic. The two broad groups are aliesterases and azolesterases, the latter exhibiting preference for nitrogen-alcohol esters (Augustinsson, 1950).

Aliesterases are subdivided into esterases that prefer short-chain aliphatic esters and lipases that prefer long-chain fatty acid esters. "True" lipase has been singled out from among the lipases on the basis of ability to hydrolyze unsaturated fatty acid esters (Gomori, 1948a).

Azolesterases include enzymes that prefer choline esters to noncholine esters and are subdivided into acetyl choline esterases, which prefer choline esters with acetic acid—among which is the cholinesterase concerned in nerve function—and cholinesterases that prefer other choline esters (Augustinsson, 1950). The functions of the latter are largely unknown; a role in limiting responses to hydrolyzable cholinergic drugs has been suggested (Karczmar and Koppanyi, 1956). The separations based on substrate affinities are relative, however, and continue to be bridged as more species and tissues are investigated (Augustinsson, 1950; Helferich, 1950).

Just as differences in substrate preferences are not absolute, so are the effects of activators and inhibitors. The effect of diagnostic agents, which have been found useful, are in a general way as stated below; but opposite effects have been obtained also, depending on species and tissue (TABLE 12).

The situation is further complicated by puzzling differences in the effects of various steps in the preincubation process, depending on species and tissue (Chessick, 1954).

In general, enzymes in the food vacuoles and cytoplasm that acted on the two acetate esters were strongly inhibited by arsenilate, while quinine was least inhibitory and frequently resulted in higher intensities. The enzymes acting on the stearate and oleate esters, on the other hand, were strongly inhibited by quinine, while arsenilate was much less inhibitory; occasional instances of higher-than-normal intensities were observed. Taurocholate, which was generally highly inhibitory with the acetate esters, was highly inhibitory,

TABLE 12*

	Esterases	Lipases	Cholinesterases
Arsenilate	Strong inhibition.	Little inhibition.	Strong inhibition.
Eserine and Prostigmine	Little inhibition.	Little inhibition.	
Fluoride	Inhibition.	Little inhibition.	
Quinine	Little inhibition; often activating.	Strong inhibition.	
Taurocholate	Strong inhibition.	Little inhibition; often activating.	

* Nachlas and Seligman, 1949a; Ammon and Jaarma, 1950; Augustinsson, 1950; Massart, 1950; Gomori, 1952a, b; Pearse, 1953; Chessick, 1954; Gomori, 1955.

especially at higher concentrations, with the long-chain fatty acid esters and, especially, in the fine cytoplasm; but higher than normal intensities were often obtained, especially in the food vacuoles and coarse deposition. The enzymes acting on myristoyl choline were outstandingly inhibited by taurocholate, quinine, and eserine, considerably inhibited by arsenilate, and not activated by taurocholate.

A separation between the enzymes acting on the acetate esters themselves appeared to be indicated by the inverse relationship between *A. dubia* and *A. proteus* and, to a lesser extent, between *P. illinoisensis* and *P. carolinensis*. The inhibitory effect of cobalt in substitution for calcium with the stearate and oleate and the lack of such a degree of inhibition with this cobalt concentration with the choline ester also suggested some difference between the enzymes demonstrated.

TABLE 13

	Substrates					Examples
	Tween 60	Tween 80	Alpha- or beta-naphthyl acetate	Naphthol AS acetate	Choline esters	
Main types						
A	+	+	—	—	—	Chief cells (mouse stomach)
B	—	—	+	+	—	Chief cells (rabbit, dog)
						Kidney tubules (human)
B ₁	—	—	+	— to ±	—	Sympathetic ganglia (several species)
						Unidentified cells, heart (rat)
B ₂	—	—	— to ±	+	—	Ampullary gland (mouse)
						Brunner's glands (rat)
						Islet cells (rat)
						Motor cells (rabbit, rat)
C	—	—	—	—	+	Astroglia (cat)
						Adrenal medulla (human)
						Certain brain cells and tracts (mouse)
						Sympathetic ganglia arborizations (several spp.)
Intermedi- ate types						
AB	+	{ + —	+ +	+ +	— —	Pancreas (rat, mouse)
						Chief cells (human stomach)
						Bronchial epithelium (several spp.)
AB ₁	+	—	+	— to ±	—	Interstitial cells (rat testis)
						Duodenal villi (rat)
AB ₂	+	—	—	+	—	Septal cells (rat lung)
AC	+	—	—	—	+	No examples known
BC	—	—	+	+	+	Spermatic elements (mouse)
						Muscle spindle (mouse)
						Conductive system (dog heart)
B ₁ C	—	—	+	—	+	Bowman capsule (dog kidney)
B ₂ C	—	—	—	+	+	No examples known
ABC	+	{ + —	+ +	+ +	+ +	Pancreas (several species)
						Liver and intestine (several species)

A = lipase; B = esterase; B₁ = alpha (or beta) esterase; B₂ = AS esterase; and C = cholinesterase.

TABLE 14

	Tween 60	Tween 80	Beta-naphthyl acetate	Naphthol AS acetate	Myristoyl choline
Food vacuoles					
<i>A.d.</i>	+	\pm	+	+	+
<i>A.p.</i>	\pm	\pm (few +)	+	\pm	\pm
<i>P.c.</i>	+	\pm	+	+	+
<i>P.i.</i>	+	\pm	+	+	+
Fine cytoplasm deposition					
<i>A.d.</i>	+ to \pm	\pm	+	+	+
<i>A.p.</i>	\pm	\pm	+	\pm	\pm
<i>P.c.</i>	\pm	\pm	+	+	+
<i>P.i.</i>	+ to \pm	\pm	+	+	+
Coarse cytoplasm deposition					
<i>A.d.</i>	+ (few)	—	—	—	+
<i>A.p.</i>	+ (rare)	—	—	—	—
<i>P.c.</i>	+	+	—	—	+
<i>P.i.</i>	+	+	—	—	+
					(sparse)
Nuclei					
All spp.	—	—	—	—	—

The Gomori classification. In a general way, there appeared to be three types of behavior with respect to diagnostic chemicals. Puzzling differences existed as to detail. Gomori concluded that although, at first glance, the results of the histochemical method appeared chaotic, reflection suggests that animal tissues contain three cardinal carboxylic esterases with narrowly defined substrate specificities and that, in addition, there are a number of enzymes of intermediate type hydrolyzing the substrates of two or even all three (TABLE 13). Whether or not these transitions actually represent individual enzymes with intermediate properties or mixtures of the pure types is as yet unresolved. The classification is tentative and no attempt was made to analyze the puzzling differences among the cholinesterases (Gomori, 1952b).

The reactions in the amoebae would be entered on such a scheme as shown in TABLE 14. From this scheme the types of carboxylic esterase activities would appear as below:

Food vacuoles. In *A. dubia*, *P. carolinensis*, and *P. illinoisensis*, activity was predominantly of the ABC type between the type characteristic of the pancreatic enzyme in several mammalian species and the hepatic and intestinal enzymes of several mammalian species.

In *A. proteus* the activity was predominantly of the B₁ type, characteristic of the ampullary gland (mouse) and certain unidentified cells in the heart (rat). Some activity at the level of the ABC type was present.

Cytoplasm giving rise to fine deposition. In *A. dubia* and in *P. illinoisensis*, activity was at a level between the ABC type as above and the BC type characteristic of muscle spindles (mouse), the conductive system in the heart (dog), and the Bowman capsule of the kidney (dog).

In *A. proteus*, activity was predominantly of the B₁ type. Some activity at the level of the ABC type was present.

In *P. carolinensis*, activity was predominantly of the BC type. Some activity on the level of the ABC type was present.

Cytoplasm giving rise to coarse deposition. In *A. dubia* activity, especially that giving rise to surface encrustation, was predominantly of the C type characteristic of adrenal medulla (human), certain cells and tracts of the brain (mouse), and arborizations around sympathetic ganglia (several species). An AC activity component characteristic of spermatocytic elements (mouse) was also present in the cytoplasm.

In *A. proteus* a "sub-A" type, for which no examples are given, was discretely dispersed.

In *P. carolinensis* and in *P. illinoisensis* the predominant characteristic was of the A type with an added C component more pronounced in *P. carolinensis*.

Nuclei. No carboxylic esterase activity was detected.

Summary

The five substrates—beta-naphthyl acetate, naphthol AS acetate, Tween 60, Tween 80, and myristoyl choline—and their laurate homologues served as microscopic enzyme cytochemical substrates for *A. dubia*, *A. proteus*, *P. carolinensis*, and *P. illinoisensis* and for the food organisms and debris in their cultures.

Primary fixation in acetone followed by drying on cover slips, rehydration with lightly hydrated acetone, and secondary drying to assure affixture, followed by secondary fixation in formalin and washing prior to incubation, resulted in the most satisfactory reactions.

Ten-grade color comparators, with intensities arithmetically graded but geometrically related, provided objectivity, reproducibility, discrimination, and information on nonspecific coloration levels. Nonspecific levels of coloration were attributable to endogenous cation and cation-insolubilizing anion, exogenous cation of the incubation and cation-exchange media, and a minimal level of coloration in the animals themselves. Heat or phenol inactivation levels integrated most nonspecific levels after demineralization procedures.

Deficiency media studies revealed that the primary reaction product with Tween 60 and with myristoyl choline, but not with Tween 80, could be trapped in the absence of precipitating cation. The explanation was based on the solubilities and melting points of the primary reaction products relative to the incubation temperatures.

Decidedly inverse relationships between *A. dubia* and *A. proteus* activities toward beta-naphthyl acetate and naphthol AS acetate suggested the duality of the corresponding enzymes. Unusually high activity toward beta-naphthyl acetate and unusually low activity toward all the other substrates in *A. proteus* suggested some compensatory mechanism. In all species, activity toward the oleate ester was lowest.

Biochemically, three cardinal types of carboxylic esterases were suggested by the effects of arsenilate, eserine, fluoride, quinine, taurocholate, cation, and substrate concentrations, and by poor reactions with intermediate chain-length homologues; pH-activity curves with lipase substrate, and the effects on them of taurocholate, bore some resemblance to those reported for lipase.

Gomori's histochemical classification of carboxylic esterases based on preferences for five substrates served as the model for the microscopic enzyme cytochemical classification of the carboxylic esterases in the amoebae.

Activity in the food vacuoles of *A. dubia*, *P. carolinensis*, and *P. illinoisensis* was predominantly of the lipase-esterase-cholinesterase type. In the food vacuoles of *A. proteus*, activity was of the alpha-esterase type, with some activity of the lipase-esterase-cholinesterase type.

In the cytoplasm, activity giving rise to fine deposition in *A. dubia* and in *P. illinoisensis* was at a level intermediate between the lipase-esterase-cholinesterase type and the esterase-cholinesterase type. In *A. proteus*, this activity was predominantly of the alpha-esterase type with some at the level of the lipase-esterase-cholinesterase type. In *P. carolinensis* activity was predominantly of the esterase-cholinesterase type, with some at the level of the lipase-esterase-cholinesterase type.

In the cytoplasm, activity giving rise to coarse deposition in *A. dubia*, especially that giving rise to more eserine-sensitive surface encrustation, was of the cholinesterase type, with some activity in the cytoplasm on the level of the lipase-cholinesterase type. In *A. proteus*, this activity was more discretely dispersed and appeared only with the stearate ester—presumably, a sublipase type. In *P. carolinensis* and *P. illinoisensis*, this activity was predominantly of the lipase type, with a cholinesterase component which was stronger in *P. carolinensis*.

The reactions in the amoebae could not be ascribed to reactions observed in the food organisms and in the debris. Debris activity toward taurocholate in the absence of Tween substrate resulted in a calcium-precipitable product. This was not observed in the amoebae.

No activity was detected in the nuclei of any of the species.

References

- ALLEN, P. J. & W. H. PRICE. 1950. The relation between respiration and protoplasmic flow in *Physarum polycephalum*. *Am. J. Botany*. **37**: 393-401.
- AMMON, R. & M. JAARMA. 1950. Enzymes hydrolyzing fats and esters. *In The Enzymes*. **1**: 390-442. Academic Press. New York, N. Y.
- ANDRESEN, N. & H. HOLTER. 1945. Cytoplasmic changes during starvation of the amoeba, *Chaos chaos* L. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **25**: 108-146.
- ANDRESEN, N. & H. HOLTER. 1949. The genera of amoebae. *Science*. **110**: 114-115.
- ANDRESEN, N., F. R. ENGEL & H. HOLTER. 1951. Succinic dehydrogenase and cytochrome oxidase in *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **27**: 408-420.
- ANDRESEN, N. & B. M. POLLOCK. 1952. Comparison between the cytoplasmic components in the myxomycete *Physarum polycephalum* in the amoeba *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **28**: 247-264.
- AUERBACH, E. 1953. A study of *Balantidium coli* in relation to cytology and behavior in culture. *J. Morphol.* **3**: 405-446.
- AUGUSTINSSON, K. B. 1950. Acetylcholinesterase and cholinesterase. *In The Enzymes*. **1**: 443-472. Academic Press. New York, N. Y.
- BARNETT, R. J. & A. M. SELIGMAN. 1951. Histochemical demonstration of esterases by the production of indigo. *Science*. **114**: 579-582.
- BARNETT, R. J. & A. M. SELIGMAN. 1954. Histochemical demonstrations of sulphydryl and disulphide groups. *J. Natl. Cancer Inst.* **14**: 769-804.
- BARRON, E. S. G. 1951. Thiol groups of biological importance. *Recent Advances in Enzymol.* **11**: 201-226.
- BERSWORTH, F. C. 1952. A powerful organic complexing agent for exacting chemical control of cations in solution. *Tech. Bull. No. 2. Bersworth Chem. Co. Framingham, Mass.*

- BÖRGSTROM, B. 1954. The effect of taurocholic acid on the pH/activity curve of pancreatic lipase. *Biochim. Biophys. Acta.* **13**: 149-151.
- BRACHET, J. 1950. Une étude cytochimique des fragments nucléés et énucléés d'amibes. *Experientia.* **6**: 294-295.
- BRACHET, J. 1951. Oxygen uptake of nucleated and non-nucleated halves of *Amoeba proteus*. *Nature.* **168**: 205.
- BRACHET, J. 1952. The role of the nucleus and the cytoplasm in synthesis and morphogenesis. Symposium Soc. Exptl. Biol. **6**: 173-200.
- BRACHET, J. 1954a. La composition enzymatique de fragments nucléés et énucléés d'amibes. *Biochim. Biophys. Acta.* **14**: 449-450.
- BRACHET, J. 1954b. Influence of the nucleus in amoeba on the breakdown of adenosine triphosphate. *Nature.* **173**: 725.
- BRANDWEIN, P. F. 1935. The culturing of fresh water protozoa and other small invertebrates. *Am. Naturalists.* **69**: 628-632.
- BUNGENBERG DE JONG, H. G. & C. BONNER. 1935. Phosphatide autocomplex coacervates as ionic systems and their relation to the protoplasmic membrane. *Protoplasma.* **24**: 198-218.
- CHESSICK, R. D. 1954. The histochemical specificity of cholinesterases. *J. Histochem. Cytochem.* **2**: 258-273.
- CLARK, A. M. 1942. Some effects of removing the nucleus from amoeba. *Australia J. Exptl. Biol. Med. Sci.* **20**: 241-243.
- CLARK, A. M. 1943. Some physiological functions of the nucleus in amoeba investigated by micrurgical methods. *Australia J. Exptl. Biol. Med. Sci.* **21**: 215-220.
- DAWSON, J. A. 1955. The culture of *Amoeba dubia*. *Biol. Rev. Coll. City N. Y.* **17**: 20-25.
- DAWSON, J. A. & M. BELKIN. 1928. The digestion of fats by *Amoeba dubia*. *Proc. Soc. Exptl. Biol. Med.* **25**: 790-793.
- DAWSON, J. A. & M. BELKIN. 1929. The digestion of oils by *Amoeba proteus*. *Biol. Bull.* **54**: 80-86.
- DOYLE, W. L. & R. LIEBELT. 1954. Diffusion of esterases after fixation. *Anat. Record.* **118**: 384.
- FOLCH, J. & M. LEES. 1950. Brain proteolipids, a new group of protein lipid substances soluble in organic solvents and not in water. *Federation Proc.* **9**: 171.
- FRENCH, D. & J. T. EDSALL. 1945. The reactions of formalin with amino acids and proteins. *Advances in Protein Chem.* **2**: 277-335.
- FROMAGEOT, C. 1952. Oxidation of organic sulphur. *In The Enzymes.* **2**: 609-623. Academic Press. New York, N. Y.
- GALBRAITH, W. 1955. The optical measurement of depth. *Quart. J. Microscop. Sci.* **96**: 285-288.
- GOMORI, G. 1948. The histochemical demonstration of sites of choline esterase activity. *Proc. Soc. Exptl. Biol. Med.* **68**: 354-358.
- GOMORI, G. 1948a. Histochemical localization of true lipase. *Proc. Soc. Exptl. Biol. Med.* **72**: 697-700.
- GOMORI, G. 1952a. *Microscopic Histochemistry.* Univ. Chicago Press. Chicago, Ill.
- GOMORI, G. 1952b. Histochemistry of esterases. *Intern. Rev. Cytol.* **1**: 323-335.
- GOMORI, G. 1955. Histochemistry of human esterases. *J. Histochem. Cytochem.* **3**: 479-484.
- GOMORI, G. & R. D. CHESSICK. 1953. Histochemical studies on the inhibition of esterases. *J. Cellular Comp. Physiol.* **41**: 51-63.
- HELFERICH, B. 1950. *The Enzymes.* **1**: 79-114. Academic Press. New York, N. Y.
- HELLER, I. 1952. Cytochemical and microincinerative studies of normal and starved *Amoeba proteus*. Ph.D. Thesis. N. Y. Univ. New York, N. Y.
- HELLER, I. M. & M. J. KOPAC. 1956. Cytochemical reactions of normal and starved *Amoeba proteus*. II. Localization of minerals. *Exptl. Cell Research.* **11**: 206-209.
- HILL, G. A. & D. KELLEY. 1943. *Organic Chemistry.* Blakiston. New York, N. Y.
- HODGMAN, C. D. & H. A. LANGE. 1930. Physical constants of organic compounds. *In Handbook of Chemistry and Physics.* Chemical Rubber. Cleveland, Ohio.
- HOLT, S. J. 1954. A new approach to the cytochemical localization of enzymes. *Proc. Roy. Soc. London.* **B142**: 160-169.
- HOLTER, H. 1949. Cell physiological work with the Amoeba *Chaos chaos*. *Proc. 6th Intern. Congr. Exptl. Cytol. Exptl. Cell Research. Suppl.* **1**: 269-276.
- HOLTER, H. 1950. The function of cell inclusions in the metabolism of *Chaos chaos*. *Ann. N. Y. Acad. Sci.* **50**(8): 1000-1009.
- HOLTER, H. 1952. Localization of enzymes in cytoplasm. *Advances in Enzymol.* **13**: 1-20.
- HOLTER, H. 1954a. Distribution of some enzymes in the cytoplasm of amoebae. *Proc. Roy. Soc. London.* **B142**: 140-146.

- HOLTER, H. 1954b. Enzymatic studies on mitochondria of amoebae. *Excerpta Med. Congr. No. 83963*.
- HOLTER, H. 1955. Fine Structure of Cells. Symposium. 8th Congr. Cell Biol. Leyden. Interscience. New York, N. Y.
- HOLTER, H. & W. L. DOYLE. 1938a. Über die Lokalisation der Amylase in Amöben. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **22**: 219-225.
- HOLTER, H. & W. L. DOYLE. 1938b. Studies on enzymatic histochemistry. *J. Cellular Compt. Physiol.* **12**: 295-308.
- HOLTER, H. & M. J. KOPAC. 1937. Studies on enzymatic histochemistry. XXIV. Localization of peptidases in the amoeba. *J. Cellular Compt. Physiol.* **10**: 423-437.
- HOLTER, H. & B. LINDERSTROM-LANGE. 1951. Micromethods and their application in the study of enzyme distribution in tissues and cells. *Physiol. Revs.* **31**: 432-448.
- HOLTER, H. & S. LÖVTRUP. 1949. Proteolytic enzymes in *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **27**: 27-62.
- HOLTER, H. & C. LUMSDEN. 1954. Cited Holter, 1954.
- HOLTER, H. & B. M. POLLOCK. 1952. Distribution of some enzymes in the cytoplasm of the myxomycete *Physarum polycephalum*. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **28**: 221-245.
- HOLTER, H. & E. ZEUTHEN. 1948. Metabolism and reduced weight in starving *Chaos chaos*. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **26**: 277-296.
- KARCZMAR, A. G. & T. KOPpanyi. 1956. Changes in transport cholinesterase levels and responses to intravenously administered acetyl choline and benzoyl choline. *J. Pharmacol. Exptl. Therap.* **116**: 245-253.
- LILLIE, R. D. 1954. Histopathologic Technique and Practical Histochemistry. Blakiston New York, N. Y.
- LINET, N. & J. BRACHET. 1951. L'évolution de l'acide ribonucléique et du glycogène dans les fragments nucléés et énucléés d'amibes. *Biochim. Biophys. Acta.* **7**: 503-696.
- LISON, L. 1953. Histochemie et Cytochimie Animales. Gautier Villars. Paris, France.
- LÖVTRUP, S. 1950. Determination of density of amoebae by means of a starch density gradient. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **26**: 137-144.
- MANHEIMER, L. H. & A. M. SELIGMAN. 1948. Improvement in the method for the histochemical demonstration of alkaline phosphatase and its use in a study of normal and neoplastic tissue. *J. Natl. Cancer Inst.* **9**: 181-199.
- MASSART, L. 1950. Enzyme inhibition. In *The Enzymes*. **1**: Academic Press. New York, N. Y.
- MAST, S. O. 1938. Digestion of fats in *Amoeba proteus*. *Biol. Bull.* **75**: 389-394.
- MAST, S. O. & W. F. HAHNERT. 1935. Feeding, digestion and starvation in *Amoeba proteus*. *L. Physiol. Zool.* **8**: 255-272.
- MAZIA, D. & H. I. HIRSHFIELD. 1950. The nucleus-dependency of P^{32} uptakes by the cell. *Science.* **112**: 297-299.
- MAZIA, D. & D. M. PRESCOTT. 1954. Nuclear function and mitosis. *Science.* **120**: 120-122.
- MAZIA, D. & D. M. PRESCOTT. 1955. The role of the nucleus in protein synthesis in amoeba. *Biochim. Biophys. Acta.* **17**: 23-34.
- MOLLER, K. M. & D. M. PRESCOTT. 1955. Observations on the cytochromes of *Amoeba proteus*, *Chaos chaos*, and *Tetrahymena geleii*. *Exptl. Cell Research.* **9**: 375-377.
- NACHLAS, M. M. & A. M. SELIGMAN. 1949. Evidence for the specificity of esterases and lipases by the use of three chromogenic substrates. *J. Biol. Chem.* **181**: 343-355.
- NACHLAS, M. M., A. C. YOUNG & A. M. SELIGMAN. 1957. The problem of enzymatic localization by chemical reactions applied to tissue sections. *J. Histochem. Cytochem.* **5**: 565-583.
- PACE, D. M. & W. H. BELDA. 1944. The effects of potassium cyanide, potassium arsenite and ethyl urethane on respiration in *Pelomyxa carolinensis*. *Biol. Bull.* **87**: 138-144.
- PACE, D. M. & B. W. McCASHLAND. 1951. Effects of low concentrations cyanide on growth and respiration in *Pelomyxa carolinensis* Wilson. *Proc. Soc. Exptl. Biol. Med.* **76**: 165-168.
- PEARSE, A. G. E. 1953. Histochemistry, Theoretical and Applied. Little, Brown. Boston, Mass.
- PEARSE, A. G. E. 1954. Azo dye methods in enzyme histochemistry. *Intern. Rev. Cytol.* **3**: 329-358.
- SELIGMAN, A. M., H. H. CHAUNCEY & M. M. NACHLAS. 1951. The effect of formalin fixation on the activity of five enzymes of rat liver. *Stain Technol.* **126**: 19-23.
- UMBREIT, W. W. 1954. Metabolic Maps. Burgess Publ. Co. Minneapolis, Minn.

- URBANI, E. 1952a. La teneur en dipeptidase et en protéinase de fragments nucléés et énucléés d'amibes. *Arch. Intern. Physiol.* **60**: 189-190.
- URBANI, E. 1952b. Sur la teneur en amylase de fragments nucléés et énucléés d'*Amoeba proteus*. *Biochim. Biophys. Acta.* **9**: 108.
- WALKER, J. F. 1953. Formaldehyde. Reinhold. New York, N. Y.
- WARD, J. M. 1958. Shift of oxidases with morphogenesis in the slime mold, *Physarum polycephalum*. *Science.* **127**: 596.
- WILBER, C. G. 1942. Digestion of fats in the rhizopod, *Pelomyxa carolinensis*. *Biol. Bull.* **83**: 320-325.
- WOLSKA, J. 1949. The small amoebae in the plasma of *Amoeba proteus*. *Ann. Univ. Mariae Curie-Skłodowska, Lublin-Polonia. Sect. C. Biol.* **4**: 137-147.
- ZACKS, S. I. 1953. Esterases in the early chick embryo. *Anat. Record.* **118**: 509-537.
- ZACKS, S. I. 1955. The cytochemistry of amoebocytes and intestinal epithelium of *Venus mercenaria*. *Quart. J. Microscop. Sci.* **96**: 57-71.
- ZEUTHEN, E. 1948a. Cartesian liver balance weighing reduced weights with an accuracy of ± 0.01 gamma. *Compt. rend. trav. Lab. Carlsberg. Sér. chim.* **26**: 243-266.
- ZEUTHEN, E. 1948b. Reduced weight and volume during starvation of the amoeba, *Chaos chaos* L. *Compt. rend. trav. Lab. Carlsberg Sér. chim.* **26**: 267-276.
- ZWEIFACH, B. W., M. M. BLACK & E. SCHORR. 1950. Histochemical alterations revealed by tetrazolium chloride in hypertensive kidneys in relation to renal v e m mechanisms. *Proc. Soc. Exptl. Biol. Med.* **74**: 848-854.

CYTOCHEMICAL DIFFERENTIATION IN NORMAL AND STARVING *AMOEBA PROTEUS*

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The term cytochemistry as used in this paper is that type of analysis in which the chemical constituents of cells are localized *in situ*. In most instances, the specific chemical constituent under investigation is visualized, directly or indirectly, as an insoluble colored compound. Microscopic observation of cellular preparations is the basis for evaluating the localization and relative concentration of a visualized chemical entity.

The review to follow includes cytochemical findings in normal and starving *Amoeba proteus* of organic constituents and of inorganic constituents. Results obtained by other methods are cited where such results are pertinent to the cytochemical findings. For the cytochemistry of anucleate and nucleate fragments the reader is referred to Brachet.^{4, 5} For cytochemical localization of enzymes in *A. proteus*, reference should be made to Guthwin's paper in this monograph.

Cytochemical Localizations in Normal A. proteus

The morphologic components of *A. proteus* are: (1) the plasmalemma; (2) the nucleus, which includes karyosomal and nucleolar components; (3) the cytoplasmic ground substance, which is microscopically free of formed bodies; and (4) the microscopically visible inclusions dispersed in the cytoplasm. The cytoplasmic inclusions are: the alpha granules, beta granules, crystals, vacuole refractive bodies, refractive bodies, fat droplets, contractile vacuole, food vacuoles, and other vacuoles. In the following sections, these morphologic components of the normal amoeba are considered in terms of their chemical composition as elucidated by cytochemical studies.

Plasmalemma. The limiting membrane of the cytoplasm was demonstrated by Pappas¹⁶ to contain: (1) a polysaccharide other than glycogen (periodic acid-Schiff positive and ptyalin resistant); and (2) tyrosine (Millon reaction) and arginine (Sakaguchi test) indicating protein. Pappas further observed that the plasmalemma did not exhibit metachromatic staining and that it was not digested by hyaluronidase. He concluded that the plasmalemma is composed in part of neutral mucopolysaccharides. Bairati and Lehmann² found that the plasmalemma reacted metachromatically in dilute toluidine blue solution and was digested by hyaluronidase. Their results suggest the presence of acid rather than neutral mucopolysaccharides.

The difference in the results of these two investigations appears to be due to the different fixatives employed. Pappas¹⁶ used formaldehyde fixation in his evaluation of metachromasia and of digestibility with hyaluronidase. Bairati and Lehmann² found that hyaluronidase attacked only the plasmalemma of amoebae that had been fixed in alcohol or acetone and not those fixed in a num-

ber of other fixatives, including formalin at several pHs. Their analyses for metachromasia were carried out on amoebae fixed in alcohol, acetone, or Bouin's fluid. The formaldehyde fixative used by Pappas¹⁶ might have resulted in the dissolution of an acid mucopolysaccharide component while leaving intact a neutral mucopolysaccharide component of the plasmalemma. An observation made by Bairati and Lehmann² strengthens this as a possibility. These investigators found that electron micrographs of the plasmalemma fixed in fluids containing acetic acid differed from those in which the fixatives used were not strongly acid. They stated that the very clear globular pattern of the plasmalemma, which is produced by acid fluids, may be due to a partial dissolution of an outer layer of mucous material, whereas less acid fixatives may preserve this layer.

Bairati and Lehmann² found that lipid solvents did not modify the structure of the plasmalemma as visualized by the electron microscope. The absence of lipids is a noteworthy observation. Hypothetical proposals of the structure of the plasma membrane, which are based in part on a consideration of permeability phenomena, generally include the presence of lipids along with proteins.

In summary, the cytochemical data indicate that the plasmalemma of *A. proteus* contains mucopolysaccharides, probably both acidic and neutral mucopolysaccharides, in association with protein. Lipid has not been demonstrated to be a component.

Karyosome. The karyosome of *A. proteus* occupies the center of the nucleus. It is an inconspicuous structure that appears as a tangled threadlike network with granules along its length. It reacts positively to the Feulgen reaction for deoxyribonucleic acids (DNA), as shown by Bogdanowicz.³ Brachet⁴ reported that the karyosome had a high concentration of arginine (Sakaguchi test) relative to that of the cytoplasm.

Nucleoli. The major portion of the nuclear volume in *A. proteus* is occupied by many peripherally placed nucleoli. The following chemical entities were determined to be in significantly higher concentrations in the nucleoli than in the cytoplasm: (1) ribonucleic acid (RNA), according to the cytochemical findings of Roskin and Ginsburg,^{17, 18} Brachet,⁴ and Heller and Kopac;⁸ (2) sulfhydryl-containing compounds, both those that are soluble as well as those that are insoluble in 10 per cent trichloroacetic acid (TCA), as demonstrated by Chalkley⁶ and Heller and Kopac,⁸ using a nitroprusside test; and (3) arginine, visualized by means of the Sakaguchi test, by Brachet.⁴ Thus, the cytochemical findings indicate that the nucleoli are rich in RNA and protein.

Heller and Kopac⁹ reported that the incinerated residues of the nucleoli of *A. proteus* appear as discrete refractile masses of high mineral concentration, presumably containing calcium or magnesium oxides, or both. This is in contrast to the fine and strikingly less concentrated ash of the cytoplasmic matrix. Heller and Kopac interpreted the ash pattern (with the exception of the mineral residue associated with excretory inclusions) as indicative of minerals associated primarily with proteins. Insofar as this interpretation is valid, the findings on microincineration support the cytochemical findings.

The quantitative data of Brachet⁵ based on chemical analyses of total RNA in anucleate and nucleate amoeba halves verify the cytochemical observations as regards the distribution of RNA in intact amoebae. Brachet determined that the initial ratio of total RNA in the nucleated amoeba fragment to that in the anucleated fragment is 1.4, as compared to the ratio of 1.1 to 1.2, which is expected if a chemical constituent is not concentrated in the nucleus. This quantitative evaluation is in good agreement with the cytochemical observation that the concentration of RNA is significantly higher in the nucleoli than in the cytoplasm.

Cytoplasmic ground substance. RNA, arginine, and sulfhydryl-containing compounds are found in the cytoplasm in concentrations that are low in comparison with the corresponding nucleolar concentrations, as indicated above. The demonstration of RNA and protein in the cytoplasm is compatible with the suggested role of the microsomes in protein synthesis, but it is in no sense proof of such association. Brachet⁴ localized tyrosine (Millon reaction) in the cytoplasm of both nucleate and anucleate fragments. Pappas¹⁶ applied the plasmal reaction in demonstrating the presence of plasmogen in the cytoplasmic ground substance.

Cytoplasmic inclusions. Fat globules present in the cytoplasm were shown by Mast and Doyle¹³ to be composed largely of neutral fat. This observation was confirmed by Pappas.¹⁶ Glycogen, while not detectable as an inclusion in the cytoplasm of living amoebae, has been demonstrated in fixed preparations by Pappas.¹⁶ The beta granules were described by Mast and Doyle¹³ as mitochondria on the basis of specific staining with Janus green, as well as by positive results obtained in using histological methods for the demonstration of mitochondria. Heller and Kopac⁸ demonstrated protein-bound sulfhydryl in these granules. Pappas¹⁶ observed positive reactions for tyrosine and arginine, and a faint coloration with Sudan black. These cytochemical findings are compatible with the description of the beta granules as mitochondria.

Mast and Doyle¹³ described two types of refractive bodies, those that are in vacuoles and those that are free in the cytoplasm. According to their analyses, the vacuole refractive bodies are homogeneous spherical bodies containing fatty acids and other lipids. The refractive bodies, which are free in the cytoplasm, were described as complex in structure. Each is composed of three morphologic components: a relatively fluid cortical layer; an inner brittle shell; and an innermost apparently fluid core. Mast and Doyle¹³ demonstrated the presence of lipids and proteins in the cortical layer. These observations were confirmed by Pappas.¹⁶ Heller and Kopac⁸ determined the presence of an organic phosphate component in the cortex of the refractive body. This organic phosphate may be responsible for the observed metachromasia of the cortex of the refractive body. Mast and Doyle¹³ considered that the inner shell of the refractive body contained carbohydrate. However, Pappas,¹⁶ using the periodic acid-Schiff test and Lugol's solution, was unable to detect either starch or glycogen in the refractive body. Heller and Kopac,^{9, 10} using cytochemical tests (the Rabl, Cretin, and Broda tests) and microincineration, demonstrated in the core of the refractive body a high concentration of calcium and magnesium, and possibly other minerals in lower concentrations. The

presence of calcium in high concentration in the refractive body has been confirmed by radioautographic studies using Ca^{45} in *A. proteus* (unpublished data).

On the basis of the reactivity of the cortical layer, Mast and Doyle¹³ and Pappas¹⁶ have suggested that the refractive bodies are Golgi bodies. The presence of a high concentration of minerals in the fluid interior of the refractive body, as shown by Heller and Kopac,^{9, 10} suggests a secretory function for the refractive body and thereby lends support to the concept that the refractive bodies are Golgi bodies. The significance of the high mineral concentration in the core of the refractive body as it relates to the metabolism of the amoeba is not understood.

Cytochemical Localizations in Starving A. proteus

Before examining the cytochemical data, it is well for purposes of orientation to consider the gross changes that occur in the living *A. proteus* during starvation. The brief description that follows is based on the reports of Mast and Hahnert,¹⁴ Andresen,¹ and Heller and Kopac.⁸ During starvation in *A. proteus* there is a progressive decrease in both the nuclear volume and the cytoplasmic volume. The rate of decrease is more pronounced in the latter and therefore, the nuclear-cytoplasmic ratio increases. The number of mitochondria decreases. The refractive bodies and crystals are maintained in number and, as a result of the volume loss, become progressively more concentrated. During the terminal stages there is a cessation of movement, followed by a rounding, and finally, by cytolysis.

Karyosome. During starvation there is a progressive condensation of the karyosome, paralleled by an increase in the over-all intensity of the Feulgen reaction, according to Brachet⁴ and Heller and Kopac.⁸ The increased intensity of the Feulgen reaction during starvation indicates either a concentration of pre-existing DNA as a result of the over-all condensation, or, an increase in the total DNA content. This question cannot be resolved on the basis of qualitative cytochemical studies and warrants further investigation.

Nucleoli. Heller and Kopac⁸ observed an increase in the size and intensity of toluidine blue-staining (indicating an increasing concentration of RNA) of the nucleoli. This increase progressed from the fifth day of starvation and reached a maximum between the ninth and the thirteenth days. Part of the progressive increase in the staining of the nucleoli was observed to be due to a progressive increase in metachromasia. Heller and Kopac interpreted this progressive increase in nucleolar metachromasia during starvation as due to some alteration in the nucleolar RNA. For example, a change in the association of RNA with protein might account for an increase in metachromasia. The inhibitory effect of proteins on metachromasia was recently reviewed by Schubert and Hamerman.¹⁹ Therefore, it is noteworthy that Heller and Kopac⁸ observed a change in protein reactivity that paralleled the changes in toluidine blue-staining of the nucleoli. These investigators observed a progressive increase in the concentration of protein-bound sulfhydryl in the nucleoli from the fifth to the thirteenth day of starvation.

Thus, the results of Heller and Kopac⁸ suggest that the following parallel

alterations in the nucleoli occur from the fifth to the thirteenth day of starvation: (1) an increase in the concentration of RNA; (2) an alteration in the structure or association of the RNA, probably related to the observed alterations in protein reactivity; and (3) an increase in the volume of the individual nucleoli. The possibility cannot be ruled out that there is a loss of total RNA and protein. Supporting this possibility is Brachet's report of a decrease in the number of nucleoli at 10 and 12 days of starvation. Brachet observed also a concomitant decrease in the basophilia of pyronine-stained nucleoli. As indicated above, Heller and Kopac noted an increasing ratio of metachromatic to orthochromatic toluidine-blue staining that reached a maximum between 9 and 13 days of starvation. In view of this, one possible interpretation of the difference in the results of Brachet⁴ and those of Heller and Kopac⁸ may be that pyronin stains only the orthochromatic and not the metachromatic component of the nucleoli. It is noteworthy that the time of maximum metachromasia, as reported by Heller and Kopac, corresponds with the period during which the decrease in pyronin basophilia was observed by Brachet.

Cytoplasmic ground substance. Heller and Kopac⁸ demonstrated that cytoplasmic RNA decreased progressively from the fifth to the thirteenth day of starvation, at which time practically no toluidine-blue coloration was detectable in the cytoplasm. Brachet,⁴ using pyronine-staining to visualize RNA, found no significant reduction in cytoplasmic RNA for a period of twelve days.

Chemical analyses for total RNA in starving amoebae have been carried out by James¹² and Brachet.⁵ James¹² found a rapid loss in total RNA in amoebae starved for 12 days. These results were confirmed by Brachet⁵ when he cultured his amoebae according to the procedure followed by James. When Brachet⁵ used his customary culturing procedure he found that total RNA remained constant in amoebae starved for 13 days. These chemical results do not appear to confirm the cytochemical results of Brachet.⁴ Since the values for total RNA are relatively constant from the first to the thirteenth day of starvation, a loss in volume (such as the loss expected during starvation) should result in an increase in RNA concentration, either in the cytoplasm, the nucleoli, or both. This is not in accord with the cytochemical findings of Brachet,⁴ that is, a constancy of the concentration of RNA in the cytoplasm during this period, and a decrease in both the number and basophilia of the nucleoli. The loss in total RNA during starvation as demonstrated by James,¹² using chemical analyses, cannot without additional information be compared to the cytochemical results for RNA in starving amoebae. In order to make a comparison between the cytochemical results for RNA that are expressed qualitatively in terms of relative concentrations with the results of chemical analyses of total RNA during starvation, it would be necessary to have the following additional information: (1) the volume changes paralleling the changes in total RNA; and (2) the contribution of the nucleolar RNA to the total RNA, both at the outset and during starvation.

Heller and Kopac⁸ applied a nitroprusside test for sulfhydryl to TCA-pre-treated whole mounts of amoebae starved from 5 to 13 days. They found a progressive increase in the protein-bound sulfhydryl in the cytoplasm and in the nucleoli (more pronounced in the latter). The maximum concentration,

reached at the thirteenth day, was observed to be maintained through 20 days of starvation.

Mazia and Prescott¹⁵ found no significant change in the rate of methionine-S³⁵ uptake by whole amoebae over a 3-day period of starvation nor any significant change in the percentage of incorporation into TCA-insoluble material during this period. These results are in general agreement with those for cytochemically determined TCA-insoluble sulfhydryl compounds where no appreciable change in concentration was noted until the fifth day of starvation.

The increased protein-bound sulfhydryl content in the cytoplasm and the nucleus of amoebae starved for 5 to 13 days could be accounted for by an increase in protein concentration. Cohen's⁷ data based on protein analyses suggest that there is no net change in total protein in *A. proteus* during 2 weeks of starvation. Since the amoebae decreased in volume during this period, it follows that an increase in protein concentration occurred. On the other hand, the results of Holter and Zeuthen¹¹ and the microincinerative studies of Heller and Kopac⁹ support the idea of a maintenance rather than an increase in protein concentration during a comparable period of starvation. Alternative explanations for the observed increase in the concentration of protein-bound sulfhydryl are: (1) a conversion during starvation of a progressively greater proportion of protein bisulfide to the reduced state; (2) preferential utilization of protein low in sulfhydryl; (3) the unmasking of protein-bound sulfhydryl resulting from the dissociation of glycogen, lipids, and nucleic acids from protein complexes; and (4) partial breakdown of the protein molecule.

Cytoplasmic inclusions. Pappas¹⁶ reported the disappearance of cytochemically demonstrated glycogen from the cytoplasm of *A. proteus* after 3 days of starvation. He found as much as one third of the normal content of lipid droplets still present in the cytoplasm of the majority of amoebae at the time of their death due to starvation. No cytochemical alterations have been noted in the alpha granules, beta granules, the plasmalemma, or the crystals of *A. proteus* during starvation. The mineral concentration of the core of the refractive body appears to remain unchanged during 22 days of starvation on the basis of the cytochemical and microincinerative results of Heller and Kopac.^{8, 9, 10} On the other hand, their cytochemical data indicate that progressive alterations occur in the cortex of the refractive body during starvation. The significance of these alterations is not clear. However, the observation of cortical changes should not, without further evidence, be considered to support the view held by Singh²⁰ and Wilber²¹ that the refractive body is reserve food.

Concluding Remarks

Perhaps the major advantage of the cytochemical approach is that it involves the localization of the chemical constituents of the cell *in situ*. Thus, morphologic cellular components, for example, nucleoli and mitochondria, while maintained intact and in their normal spatial relationships, are defined in terms of their chemical composition. Furthermore, the cytochemical analysis of experimentally modified cells readily yields results that are obtained only with difficulty by other methods, for it is possible to visualize the alterations in the localization and concentration of chemical entities occurring simultaneously in

the various cellular components, for example, an increase in RNA in the nucleoli and a concomitant decrease in RNA in the cytoplasm. These advantages of the cytochemical approach are exemplified by the data reviewed above.

The disadvantages of the cytochemical approach are those intrinsic in the available procedures, which do not always fulfill the following criteria for excellence: (1) specificity; (2) high sensitivity; (3) absence of diffusion; and (4) suitability for quantitation. It is essential in this, as in all methodology, to be cognizant of the limitations of the techniques used and to seek confirmation from some other independent type of analysis.

The applications of cytochemistry to the analysis of the chemical composition of normal and experimentally altered *A. proteus* have been fruitful. The further use of cytochemistry can enrich the understanding of both the structure and function of *A. proteus*. Quantitative cytochemistry should be applied to the study of *A. proteus* whenever it is possible and desirable. Confirmation of the data presented above should be sought both by the use of such improved cytochemical methods as are available and by the use of other methods.

References

1. ANDRESEN, N. 1945. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **25**: 169.
2. BAIRATI, A. & F. E. LEHMANN. 1953. Exptl. Cell Research. **5**: 220.
3. BOGDANOWICZ, A. 1930. Z. Zellforsch. Mikroskop. Anat. **10**: 471.
4. BRACHET, J. 1950. Experientia. **6**: 294.
5. BRACHET, J. 1955. Biochim. Biophys. Acta. **18**: 247.
6. CHALKLEY, H. W. 1937. Protoplasma. **28**: 489.
7. COHEN, A. I. 1957. J. Biophys. Biochem. Cytol. **3**: 923.
8. HELLER, I. M. & M. J. KOPAC. 1955. Exptl. Cell Research. **8**: 62.
9. HELLER, I. M. & M. J. KOPAC. 1955. Exptl. Cell Research. **8**: 563.
10. HELLER, I. M. & M. J. KOPAC. 1956. Exptl. Cell Research. **11**: 206.
11. HOLTER, H. & E. ZUETHEN. 1948. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **26**: 227.
12. JAMES, T. W. 1954. Biochim. Biophys. Acta. **15**: 367.
13. MAST, S. O. & W. L. DOYLE. 1935. Arch. Protistenk. **86**: 155.
14. MAST, S. O. & W. F. HAHNERT. 1935. Physiol. Zool. **8**: 255.
15. MAZIA, D. & D. M. PRESCOTT. 1955. Biochim. Biophys. Acta. **17**: 23.
16. PAPPAS, G. 1954. Ohio J. Sci. **54**: 195.
17. ROSKIN, G. I. & A. S. GINSBURG. 1944. Compt. rend. acad. sci. U.R.S.S. **42**: 348.
18. ROSKIN, G. I. & A. S. GINSBURG. 1944. Compt. rend. acad. sci. U.R.S.S. **43**: 122.
19. SCHUBERT, M. & D. HAMERMAN. 1956. J. Histochem. Cytochem. **4**: 158.
20. SINGH, B. N. 1938. Quart. J. Microscop. Sci. **80**: 601.
21. WILBER, C. G. 1945. Biol. Bull. **88**: 207.

PHYSIOLOGICAL AND MORPHOLOGICAL OBSERVATIONS ON AMOEBAE*

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One of the cherished goals of cell physiology is to understand the relationships among the activities that are compartmentalized within the cellular organelles. Toward this end, cell inanition constitutes a procedure that may be of considerable value inasmuch as it may enhance a competition for various substances. These competitions form the basis for concepts regarding the mechanisms of physiological phenomena such as the Pasteur effect.^{1, 2, 3} Cellular inanition may also reveal information about such phenomena as molecular stability and turnover, inasmuch as the dynamic turnover of certain molecules would appear to be a process dependent upon energy.^{4, 5} Finally, such studies may bear on the mode of activation of those autolytic changes that might be classed as degradations not requiring energy. Although a considerable literature exists on starvation effects exhibited by mammalian tissues,⁶ these inherently suffer from the facts that organs contain mixed cell populations, that the cell environment is both unknown and probably changing, and that the cells cannot be proved to be under total inanition, since it is only the total organism that is being deprived of foodstuffs.

With regard to microorganisms, the classic studies of A. I. Virtanen and his group⁷ on bacteria under inanition have shown that, relative to nitrogen, certain enzymes remain constant, others show relative increases, and still others decrease in activity as the population starves. While of immense value, these experiments on bacterial populations deal with chemoautotrophic nutrition and with cells, the cytological compartments of which remain to be defined.

In the free-living amoebae, on the other hand, a cell is available, the starvation of which may be controlled by the most precise regulation of the external medium. Moreover, it is highly probable that the nutrition is of the heterotrophic type. Finally, light and electron microscopic studies have revealed a cytological picture that, although possessing some unique aspects is, on the whole, quite typical of that exhibited by cells in general.^{8, 9}

Starvation effects on amoebae have been studied with the light microscope by Mast and Hahnert¹⁰ and by Andresen¹¹ and, using cytochemical methods, by Brachet^{12, 13} and by Heller and Kopac.¹⁴ Information of a chemical nature has been obtained directly¹⁵ and also deduced from certain indirect physiological measurements.¹⁶ Frequently starvation data have been made available from investigations in which comparisons of nucleate and enucleate amoeba cell portions have been reported. Inasmuch as enucleate amoeba do not feed, control animals must be kept under starvation. This work has been summarized by Brachet.¹³

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The data to be presented herewith relate to the use of inanition and other methods to explore the aforementioned areas.

Materials and Methods

The race of amoeba employed in these studies was obtained from the Chicago Biological Supply House and differs from other races of *A. proteus* in its size, marked light sensitivity, and ability to feed on the ciliate *Tetrahymena*. Cultures were made in agar-free finger bowls, using a modified Hahnert's medium, as described by Prescott.¹⁷

Starving populations were transferred daily to fresh medium and counted during the transfer process.

DNA was assayed fluorimetrically by a new method of Kissane and Robins³¹ and DNA studies were performed in collaboration with Kissane. This method is very sensitive to desoxyribose, and the amoebae and ciliates assayed were freed of free-desoxyribose compounds and lipid by exhaustive extractions. Accordingly, highly polymerized DNA was the only reactive material known to be present.

Labeling the amoebae with radioisotopes was effected by labeling the food organisms prior to feeding them to the amoebae. After feeding on the radioactive food organisms, amoebae were washed and allowed to feed on unlabeled food for one or two days prior to experimentation. At this time they were transferred to a small Petri dish and given a slight excess of food. Since heavily fed cells do not migrate, after 24 hours it was possible to recover sister pairs of cells. One of these cells was then allowed to dry down on a planchette for radioassay, while the corresponding sister cell was fed or starved, as the experiment required. A nuclear D-46A Q-gas counter was employed for detection of S^{35} radiation and a Geiger tube for P^{32} radiation. For assaying living amoebae labeled with P^{32} a small chamber with a thin latex sheet floor was placed on the window of a light-shielded, vertically mounted Geiger-Muller tube and the cell, in a droplet of known volume, was placed in a marked area and allowed to settle before counting.

Electron microscopic observations were made of fed and starved animals. These amoebae were fixed in Dalton's chrome-osmic fixative¹⁸ and embedded in an agar block. This block was then dehydrated and embedded in methacrylate and sectioned on a Porter-Blum microtome. Thick sections were observed in a phase microscope for orientation and contiguous thin sections observed in an RCA 2-E electron microscope.

Experimental

One of the first questions to be considered is whether there is protein turnover in growing populations. Studies by Hogness *et al.*¹⁹ on *Escherichia coli* have suggested that labeled protein did not turn over and that its concentration fell on a unit nitrogen basis only through dilution as the mass of protoplasm and the cell number increased.

Tetrahymena were raised in 2 per cent peptone broth containing 500 μ c. of S^{35} -labeled methionine in 50 ml. and harvested after 5 days of growth. These

TABLE 1
THE DISTRIBUTION OF S³⁵ ACTIVITY FOLLOWING CELL DIVISION

Pair	Total activity (cpm/cell)		Pair	Total activity (cpm/cell)	
1	28.9	43.8	9	43.8	33.8
2	32.5	31.2	10	37.6	34.3
3	19.5	23.7	11	85.1	70.6
4	15.0	17.0	12	131.0	97.0
5	35.3	19.0	13	79.5	68.0
6	19.2	21.5	14	109.5	71.5
7	34.2	47.9	15	57.5	56.0
8	47.9	30.3			

organisms were fed to amoebae for 4 days, followed by feeding on isotope-free food, as previously described. When sister cells were assayed for radioactivity, as TABLE 1 shows, considerable differences may exist between the activities of the sister cells, although a distinct tendency is evident for the two cells to be more alike than are cells selected at random.

Does the level of disparity between sister cells reflect unequal division, or is it due to other causes? The data of Prescott²⁰ on the reduced weights of sister cells show that, if undisturbed during division, as were the cells mentioned above, excellent weight correspondence exists between sister cells. The suggestion is then clear that there exist, in the cytoplasm of the amoeba, foci of radioactivity so small in number and so active that randomness of distribution at division may yield substantial activity differences. Electron microscopy has shown the persistence of *Tetrahymena* residues in amoeba cytoplasm for weeks under starvation, but it is not easy to decide if a particular vacuole in a feeding cell is a new or an old one. Some preliminary data have been obtained by using *Tetrahymena* tagged by ingested colloidal gold that were fed to amoebae (FIGURE 1). The second generation descendants of these cells, raised on untagged food, were examined in the electron microscope and have shown that some colloidal gold vacuoles persist (FIGURE 2). No quantitative estimates can be made. The use of radioactive colloidal gold, if not injurious to the cells, may permit such quantitative estimation.

Andresen *et al.*³⁸ fed C¹⁴-labeled food organisms to individuals of the multi-nucleate species *Chaos chaos* and observed the fate of the radioactivity during subsequent feeding on unlabeled food. They found a period of acute reduction of activity (defecation) but with persistence of activity as long as 700 to 800 hours. Centrifugal stratification of the amoebae followed by radioautography revealed the persistence of activity in vacuoles that did not contain newly ingested food. Accordingly, the above study using colloidal gold confirms Andresen's conclusion that food derivatives persist in vacuoles for some time after the original feeding. Colloidal gold particles are readily distinguished from foamy particles and granular material occasionally remaining around the holes left by the amoeba crystals that disappear at some stage in electron-microscope procedure (FIGURE 3).

Returning to the fate of sulfur labels in feeding populations, TABLE 2 depicts

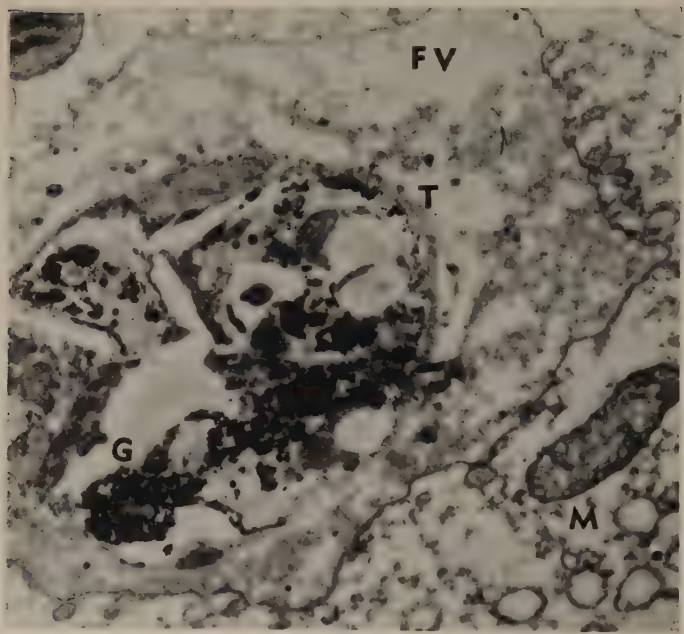


FIGURE 1

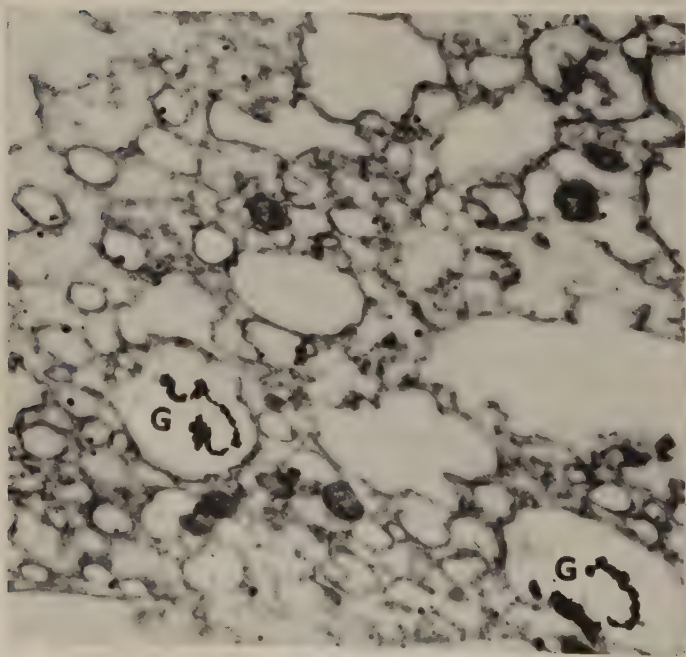


FIGURE 2

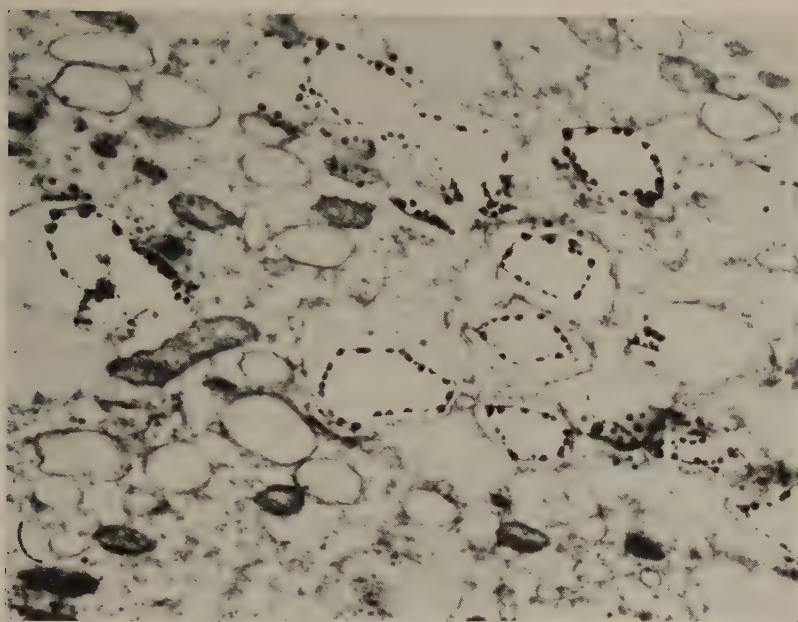


FIGURE 3

TABLE 2

THE REDISTRIBUTION OF S^{35} LABEL WITH THE MULTIPLICATION OF AMOEBAE

Expt.	No. cells	Total activity (cpm)		Loss (percentages)
		Before TCA	After TCA†	
1A	1	672	352	47.6
B*	21	326	231	26.0
2A	1	474	288	39.2
B	28	301	269	10.6
3A	1	403	193	52.1
B	43	198	135	31.8
4A	1	307	167	45.8
B	21	154	115	25.3
5A	1	455	275	39.5
B	39	288	180	37.5
6A	1	358	245	31.5
B	83	278	233	16.2
7A	1	397	293	26.2
B	70	333	286	20.2
8A	1	301	261	13.3
B	51	198	140	29.3
9A	1	352	248	29.5
B	50	160	120	25.0
10A	1	615	383	37.8
B	31	294	223	24.1

* B refers to cells resulting from the multiplication of a sister cell of A.

† Five per cent cold TCA for 1 hour.

an experiment in which cells were fed on S^{35} -labeled *Chilomonas* for 2 days and then on unlabeled *Tetrahymena* for 1 day. At this time sister cell pairs were collected and 1 cell of each pair was used for assay. The other cell was allowed to feed on unlabeled food and the total population resulting from each cell after 6 days at room temperature (circa 25° C.) was assayed as a unit after mounting on a single planchette. The single cell and the population resulting from its sister cell were then extracted with 5 per cent trichloroacetic acid for 1 hour and reassayed.

The results show that all feeding populations assayed a lower total activity both before and after TCA extraction than did the sister cell mounted at zero time. Eight of the ten resulting populations showed a marked increase in the per cent of activity in the non-TCA extractable fraction, presumably protein. One population showed a nonsignificant percentile increase and one population a very marked percentile decrease. The results show that label loss was more extensive from the TCA extractable fraction than from the nonextractable fraction, although both lost label under conditions of growth. Whether this is to be interpreted as suggesting amoeba protein turnover depends on the fate of label in the food protein and the persistence and mode of digestion of food protein in the cytoplasm.

When a similar experiment was performed, with the labeled amoebae allowed to feed for a longer time on unlabeled food before the sister cells were selected (that is, 48 hours on unlabeled food instead of 24 hours), then the percentile losses from both the TCA extractable and nonextractable fractions were about the same and very much reduced in absolute amounts. This suggests that, when there is a considerable residue of labeled food in the cytoplasm, the free amino acid pool would be somewhat richer in label than the total protein (food protein plus amoeba protein), and a substantial part of the total loss must occur early in the experiment.

Studies comparable to the feeding experiments were performed with starving amoebae and suggested little label loss and greater incorporation into the TCA nonextractable fraction. However, a more direct attack can be made on starvation protein levels by direct measurement. This work, previously reported¹⁵ showed that protein was eventually lost during terminal starvation, but that the net level was surprisingly stable for a long time. A comparison of the changes in the dry weight of lyophilized cells during starvation and measurements of a fraction extractable with hexane and alcohol suggest that carbohydrate and lipid are responsible for most of the early weight loss. This confirms deductions made from Cartesian diver observations by Holter and Zeuthen¹⁶ and fits the pattern of behavior of nucleate amoeba fragments as reported by Brachet.¹³

These results suggest a general stability on the part of structural protein during starvation and support the concept that nonenergy-requiring degradation of certain molecular species may not be a continuing metabolic feature in some sort of balance with synthetic activity.

Because there is some disagreement as to the stability of ribonucleic acid in whole amoebae during starvation,^{13, 14, 21} it was decided to see whether starving cells lose considerable phosphate label. It had already been demonstrated by

TABLE 3
THE P³² LEVEL OF LIVING AMOEBAE DURING STARVATION (PAIRS)

Day	Solution				
	A	B	C	D	E
0	625	449	498	424	514
1	417	605	349	422	409
2	590	500	365	468	450
3	485	538	480	572	485
4	636	610	430	426	636
5	670	600	570	500	670
6	610	585	565	525	610
7	572	480	450	450	572
8	565	535	488	440	—
9	515	555	415	230 (½)	—
10	642	267 (½)	255 (½)	—	—

Solutions: A, sodium glycerophosphate 0.004 M in amoeba medium; B, phosphate-free medium, no additives; C, phosphate-free medium, 0.004 glucose; D, amoeba medium; and E, amoeba medium, 0.004 glucose.

Friedrich-Freksa and Kaudewitz²² that feeding amoebae show no detectable loss of previously incorporated label except by dilution through growth. Cells were labeled through feeding P³²-labeled *Tetrahymena* or *Chilomonas*, allowed to feed for two days on unlabeled food organisms, and then assayed while alive and starving. If, during early starvation, a cell divided, the pair were then carried forward as a unit and assayed together. Although it is doubtful that amoebae are permeable to glucose or glycerophosphate, starvation was also followed in the presence of these materials and in the presence and absence of phosphate ion. Finally, feeding cells were followed.

The results, shown in TABLE 3, indicate no gross label loss under any circumstance. It may be that breakdown products do not escape from the cell. The short life span under starvation in this experiment is due to the fact that the cells were kept at a temperature close to 25° C. The sensitivity of the method is indicated by the change in reading on the terminal days when one of the pair of cells survived longer than its sister cell.

Attempts were made to discern the biochemical distribution of P³² in labeled animals by treating the organisms, fixed on planchettes, with such agents as perchloric acid and ribonuclease, and then reassaying, but these procedures have proved unreliable.

As it is not known what fraction of the total phosphate of an amoeba was represented by ribonucleic acid, the results, although best fitting the concept of net stability during starvation, are not very revealing.

Another entity whose stability has been studied under conditions of early starvation is diphosphopyridine nucleotide. These studies have been motivated by the finding that an enzyme for synthesizing DPN has been found in the nuclei of liver cells,²³ nucleated erythrocytes,²⁴ and the nucleoli of starfish oocytes.²⁵ The question then arose whether the rapidly appearing (minutes) symptoms of enucleation might be attributable to a fall in the level of this important metabolite. Results of studies comparing nucleate and enucleate

fragments have been simultaneously reported by Baltus²⁶ and by ourselves.²⁷ Curiously, while reporting essentially the same DPN levels and DPN stability (for one week) in unfed nucleate animals, in Baltus' hands a rapid loss of DPN was found in enucleate animals. We, on the other hand, could detect no loss by a very much more sensitive method. It should be noted that the disparities for DPN in her nucleate and enucleate fragments are no greater than those reported for protein by the same laboratory in two other investigations covering similar time intervals.^{13, 28} Enucleate amoebae perhaps resemble enucleate erythrocytes that maintain their DPN level over their long life span in the absence of a nucleus²⁹ and despite a lack of DPN-synthesizing enzyme,²⁴ possibly because of a confinement of DPNase to the erythrocyte membrane.³⁰

In all the studies discussed thus far, one of the great obstacles to meaningful quantitation was that of distinguishing vacuolar contents from extravacuolar cytoplasm. This distinction is particularly pertinent when one deals with cells engaged in active pinocytosis (such as exhibited by *Tetrahymena* in peptone broth) or phagocytosis.

In the case of amoebae, for example, the use of nutritive materials involves first ingestion, then digestion and, finally, assimilation. One may ask the following questions. How much of an amoeba at any stage of growth is food vacuole? Does the number of food vacuoles influence feeding activity? If this is so, what effect does the rate of feeding and percentage of animal that is food vacuole have on growth curves based on mass, volume, or protein? Are there differences in the rate of digestion of vacuoles in amoebae that are feeding as opposed to those cut off from additional food? It is obvious that any source of food never provides the cell with exactly what it requires, in the sense that some substances will be in excess of need and some material may be relatively indigestible. What is the fate of these materials? To what extent do they remain in the cell? Finally, if one notes a difference in the content of a given substance in enucleate animals, are we noting a defect of digestion or of subsequent metabolism?

With the advent of a technique for mass amoeba culture on *Tetrahymena*¹⁷ and with the development of a fluorimetric DNA assay method³¹ there has appeared an inroad to the solution of some of the above-mentioned problems. On the reasonable assumptions that the DNA that is genetically amoeba is confined to the amoeba nucleus and that any cytoplasmic DNA represents that of food organisms, if one knows the value for the DNA of a *Tetrahymena*, one can interpret the level of cytoplasmic DNA in an amoeba in terms of the number of *Tetrahymena* this figure represents.

Of course, it is not to be expected that all the macromolecular species of a food organism will be digested at the same rate. It is likewise not to be expected that the qualitative and quantitative abstraction by amoeba synthetic mechanisms of building blocks from the pools of various residues will proceed at a uniform rate with reference to the interdivision cycle of the amoeba. However, these are problems for the future. For the present, therefore, we shall make the simplifying assumptions that the digestion of cytoplasmic DNA is a valid first-order approximation of the digestion of other food macromole-

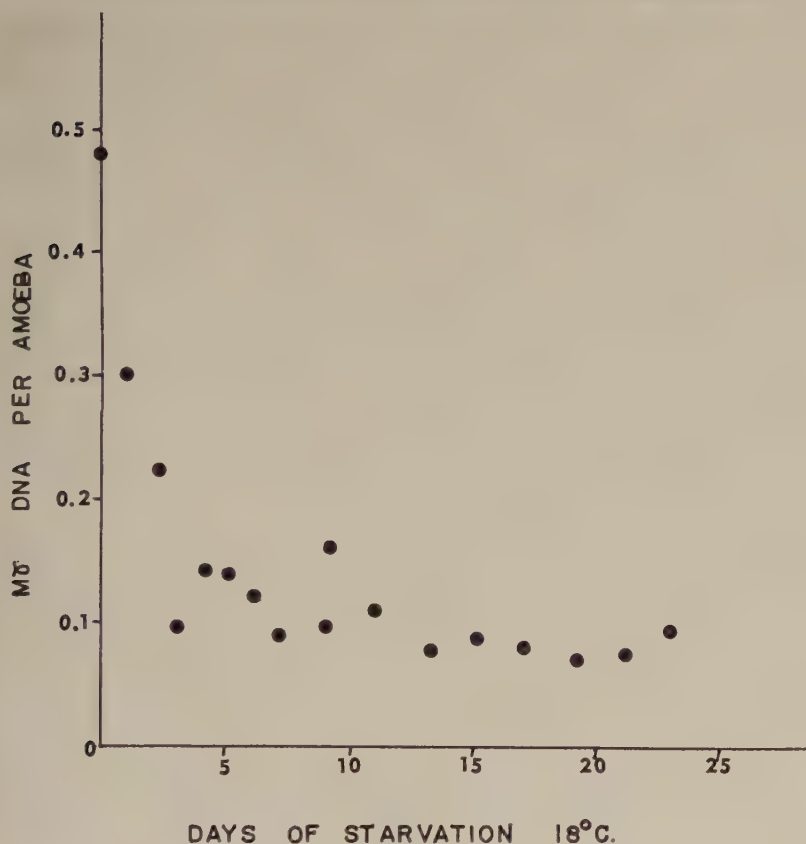


FIGURE 4

cules and that, with regard to the interdivision cycle, the wants of the amoeba are unchanging.

FIGURE 4 shows the fall of DNA per amoeba as a function of days of starvation. Each determination represents a value obtained from 400 cells, except for the initial point, representing 250 heavily fed animals. The steepness of the initial decline in the level per cell is somewhat exaggerated if viewed as a digestion rate because, during the first week of starvation, cells may divide. The final plateau reached then represents the level of amoeba DNA and a possible contribution of indigestible, retained food DNA.

From a starting level of 0.48 $\mu\text{g.}$ of DNA per cell at 18° C. about 40 per cent disappears in the first 24 hours, about 13 per cent in the second 24-hour period, about 6 per cent in the third 24-hour period, and the DNA level eventually settles down to about 0.08 $\mu\text{g.}$ in about 12 days (FIGURE 4). Individual *Tetrahymena* were picked up with a braking pipette and a standard curve determined for DNA versus number of cells. DNA levels of 5.58, 10.9, and 21.2 $\mu\text{g.}$ were obtained for 200, 400, and 800 animals, respectively. This

calculates to a level of 0.027 $\mu\text{g.}$ per cell and shows that the chemistry is sensitive to the DNA of one fourth of a *Tetrahymena* per amoeba in a sample of 400 amoebae. Since the difference between the initial and final amoeba levels was 0.40 $\mu\text{g.}$, about 15 *Tetrahymena* can account for this decrease. Iverson and Giese³² found 0.030 $\mu\text{g.}$ of DNA per *Tetrahymena*, and Scherbaum³³ found 0.013 $\mu\text{g.}$ per *Tetrahymena*. Our findings, therefore, support those of Iverson and Giese, although all reports are of the same order of magnitude.

A random sample of 25 well-fed amoebae, fixed with Carnoy's and stained by the Feulgen-fast green method, gave an average of 45.2 fast green-staining vacuoles per amoeba (median = 44), of which an average of 17.1 vacuoles per amoeba (median = 14) had Feulgen-positive material visually evident within the vacuole.

Scherbaum reports³³ that the dry weight of *Tetrahymena* is 4.1 $\mu\text{g.}$ James and Read³⁹ give a value for volume of $12.3 \times 10^3 \mu^3$ for a *Tetrahymena* at 20° C. As the mean dry weight¹⁵ of an amoeba is 0.23 $\mu\text{g.}$ and its volume¹⁵ is $14.8 \times 10^3 \mu^3$, fifteen ingested food organisms constitute 25 per cent of the amoeba mass and 12 per cent of the volume.

Many are curious to know how much of the RNA contained in the amoeba is *Tetrahymena* RNA. Unfortunately, the figures for *Tetrahymena* RNA given by Iverson and Giese³² and by Scherbaum³³ differ considerably, the latter reporting $24.6 \times 10^{-5} \mu\text{g.}$ per cell and the former giving $1.28 \times 10^{-3} \mu\text{g.}$ per cell. The figures given for the total RNA of an amoeba likewise differ, James²¹ giving 0.100 $\mu\text{g.}$ per 100 amoebae and Brachet¹³ reporting about 0.68 $\mu\text{g.}$ per 100 whole animals. Using the smaller value for *Tetrahymena* RNA, 15 animals have $369 \times 10^{-5} \mu\text{g.}$ of RNA. Using the larger value for amoebae, namely 0.68 $\mu\text{g.}$ per 100 cells, one animal has $680 \times 10^{-5} \mu\text{g.}$ Therefore, 54 per cent of the initial amoeba RNA level calculates to be RNA contained in food vacuoles!

Granting certain difficulties in RNA determinations, it nevertheless follows that the well-nourished amoeba may well have a very considerable percentage of its total RNA content within food vacuoles. One is then led to a reconsideration of certain reports relating to RNA level.

RNA exists in 3 amoeba compartments: namely, nuclear, cytoplasmic, and vacuolar. It has been demonstrated that at least some cytoplasmic RNA derives from the nucleus.³⁴ It has also been demonstrated, by means of radioautography, that in feeding cells the loss of nuclear RNA label is far more rapid than in starving cells,³⁵ thus suggesting a dependency of nuclear RNA turnover on available energy. Cytochemical RNA observations by Heller and Kopac¹⁴ suggest a slight increase in cytoplasmic RNA concentration followed by a gradual fall in RNA concentration during starvation, with no toluidine-blue staining evident in 13 days at 18 to 20° C. These investigators suggest that pyronine B, employed by Brachet³⁶ in a similar study in which no decrease in basophilia occurred in starvation, is not specific because it stains other materials that are progressively increasing, concomitantly with RNA disappearance. However, Brachet also employed ribonuclease as a control, so this argument

does not seem valid. The answer may lie in different dye-binding capacities, thus making one dye a more sensitive RNA indicator than another.

It then becomes apparent that the nutritional level of the amoeba will strongly influence RNA levels and their changes in starvation. If there is no net increase in amoeba RNA, then the digestion of food in itself can account for a fall of 50 per cent of the total RNA. It seems reasonable to suggest that, where no RNA loss has been observed in nucleate half amoebae, the whole animals had little in the way of food reserves. Thus James²¹ raw data show a loss of 56 per cent of the RNA of whole animals and a loss of 40 per cent of the RNA nucleate half animals in the same time interval. Brachet¹³ found a loss of only 20 per cent of the RNA of whole animals and no loss for nucleate halves. This also suggests a relative stability for the RNA intrinsic to the amoeba, as opposed to food RNA, during early starvation. Turning to the enucleate animal, we may expect a continuation of food digestion in progress at the time of operation. In addition, inasmuch as this fragment has a shortened preautolytic life span as compared to starving whole cells and nucleate halves,¹⁵ the beginnings of autolysis will occur within the period when nucleate cells are relatively stable. If this autolysis involves amoeba-ribonucleoprotein destruction, then the loss of RNA in the enucleate animal will involve digestion of food, the absence of any RNA replacement from the nucleus, and autolysis of amoeba ribonucleoprotein. Both James and Brachet find massive RNA losses (70 per cent and 60 per cent) in enucleates. However, inasmuch as their reported absolute RNA levels differed sharply, it would be hazardous to attempt to attribute these losses to specific compartments. It is also evident that amoebae feeding on *Tetrahymena* will accumulate considerable excesses of DNA and RNA above any reasonable need for purine and pyrimidine nucleotides. This requires some system of disposal and also poses an experimental variable since externally applied radionucleotides will be variably diluted in proportion to vacuole content.

The interpretation of curves drawn to express the mass of amoeba during growth or starvation is obviously complicated by the fact that 25 per cent of the mass may be food-vacuole content. This complication not only applies to amoebae, but also to cells feeding by pinocytosis, as the massive uptake of colloidal gold by *Tetrahymena* grown in peptone broth indicates. Some sort of serologic approach might reveal the true pattern of the increment in uniquely amoeba antigens between divisions.

As an example of the difficulty encountered, the progressive increase in mass of a growing amoeba ceases some hours before division ensues.³⁷ Is this simply a cessation of feeding? It is quite possible that previously ingested food materials are still being digested and converted to amoeba molecules.

Figures for *Tetrahymena* protein per cell are not yet available and, for this reason, we cannot state the extent to which such protein contaminates feeding amoebae. One way of following *Tetrahymena* protein in feeding and starving cells would be as follows. One could raise amoebae for a number of generations with the flagellate *Chilomonas* as a food source. In the meantime, an antiserum would be prepared to *Tetrahymena* protein. Such an antiserum would

be treated with amoeba protein and flagellate protein to remove antibodies common to antigens of *Tetrahymena* and these organisms. The remaining antiserum would then respond to whatever uniquely *Tetrahymena* protein antigens were most antigenic (since not all proteins are equally antigenic) and would be a useful reagent to detect the presence of these materials in feeding and starving cells raised on *Tetrahymena*.

Existing data on starving animals¹⁵ show no net protein change until terminal starvation. However, it seems reasonable to conclude that protein digestion is proceeding. If 25 per cent of the original amoeba protein level is, in fact, *Tetrahymena* protein, then most of this must be converted to amoeba protein with fair efficiency, since there is no net protein change and since preliminary isotope data show little shift in the total label (S^{35}) or in TCA nonextractable label during the first week of starvation.

This suggests that, if the accumulation of amoeba molecules be the criterion of growth, then the amoeba is growing during early starvation, its growth masked by food digestion. This idea is supported by the persistence of cell divisions during the first week of starvation.^{15, 37}

Discussion

The experiments in this paper indicate that, although the amoeba has unique advantages as a tool in cell research, it likewise possesses a number of complicating factors relating to phagocytic feeding. These same factors will also apply, although perhaps to a lesser extent, to cells such as *Tetrahymena* in peptone broth that are engaging in pinocytic feeding. It becomes apparent that the term intracellular possesses an important degree of vagueness: namely, whether vacuolar compartments that are, grossly considered, within the cell may be considered, from certain physiological points of view, outside the cytoplasm. Although the vacuolar membrane may be regarded as a derivative of the cell membrane, it is not unlikely that certain permeability modifications may ensue in the course of processing enclosed material.

In considering the literature on amoebae, one cannot help but feel that progress in our understanding of the complexities of the physiology of this cell would be greatly enhanced if all investigators would pay more attention to specifying in their publications such essential points of information as the nature of culturing, including such factors as food, salt medium, presence or absence of agar, temperature, and frequency of medium change. Without this information, comparisons of data become most precarious.

Another point to be noted is that *A. proteus*, in our experience, consists of at least two strains that are distinct in certain physiological ways. The problem of setting a useful definition of species for asexual organisms is a most difficult one. Accordingly, investigators should describe, if possible, the history of their amoeba stock.

In performing experiments on enucleate cells, it is important that some effort be made to standardize the nutrition of the amoeba before experimentation. It may well be that maximal feeding followed by a deliberate and known interval of starvation before enucleation will tend to make nuclear activities

easier to detect and will eliminate some of the confusing variability in certain investigations.

Along the same lines, we have noted that the detection of enucleate fragments is not always a simple matter. Using the Chicago Biological Supply clone of amoeba, we have had great success in using photophobia as a means of driving nucleate and relatively motile organisms from the feebly moving enucleates. This has been useful in determinations where chemical insensitivity precludes the use of small numbers of cells in which the presence of the nucleus can be checked microscopically. On the other hand, we have had poor success in using the same method with an *A. proteus* obtained from other sources or with amoeba halves that have been prepared after a deliberate period of starvation. In this case, some nucleate halves are relatively immobile.

Accordingly, the practice of deciding that a cell is enucleate because it is rounded up and immobile is fraught with danger. Whatever system is employed, samples should be obtained to be checked for the efficacy of separation by staining (mercuric-bromphenol blue is useful) for the presence of nuclei.

Summary

A number of approaches to the study of the turnover of various molecular fractions in growing and starving amoebae have been described.

Starving amoebae show little loss of previously incorporated food radio-sulfate from either TCA extractable or nonextractable fractions. Net protein levels remain stable until terminal starvation.

Feeding amoebae show label loss from both the TCA extractable and non-extractable fractions, but the amount of loss and relative loss from each fraction appears to be conditioned by the presence of radioactive food residues. Preliminary evidence suggests markedly diminished loss from both fractions with continued growth and multiplication, using unlabeled food.

After feeding *Tetrahymena* containing colloidal gold to amoebae, electron microscopy shows that gold is observable for two generations of growth on gold-free food. The suggestion that food remnants may persist for such periods complicates the interpretation of the isotope data.

Radiophosphate from labeled food was crudely assayed in living amoebae during starvation, and the level was found to remain stable.

Using a fluorimetric DNA method, DNA was assayed in well-fed amoebae and during starvation. The level fell from 0.48 μg . per cell to a plateau value of 0.08 μg . per cell after 12 days at 18° C. This decrease corresponds to the DNA of 15 food organisms (*Tetrahymena*) and shows that in the average well-fed amoeba food vacuoles are a considerable contaminant. A study of the literature shows that 15 *Tetrahymena* may constitute 12 per cent of the volume, 25 per cent of the total mass, and 50 per cent of the total RNA of the amoeba.

The physiology of the amoeba is discussed with reference to the above-mentioned data. The extent of food contamination renders the interpretation of mass changes during growth and starvation difficult if it is intended to follow the levels of amoeba molecules. Variation in feeding levels may also account for certain disagreements of data from various laboratories.

References

1. LYNEN, F. 1941. *Ann. Chem. Liebigs.* **546**: 120.
2. JOHNSON, M. F. 1941. *Science.* **94**: 200.
3. KREBS, H. A. 1957. *Endeavour.* **125**.
4. SIMPSON, M. V. 1953. *J. Biol. Chem.* **201**: 143.
5. STEINBERG, D. & M. VAUGHAN. 1956. *Arch. Biochem. Biophys.* **65**: 93.
6. WAINIO, W., B. EICHEL, H. EICHEL, P. PEARSON, F. L. ESTES & J. B. ALLISON. 1953. *J. Nutrition.* **49**: 465.
7. VIRTANEN, A. I. 1952. *Ann. Med. Exptl. et Biol. Fenniae (Helsinki).* **30**: 234.
8. ANDRESEN, N. 1956. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **29**: 435.
9. COHEN, A. I. 1957. *J. Biophys. Biochem. Cytol.* **3**: 859.
10. MAST, S. O. & W. F. HAHNERT. 1935. *Physiol. Zool.* **8**: 255.
11. ANDRESEN, N. 1945. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **25**: 169.
12. BRACHET, J. 1950. *Experientia.* **6**: 294.
13. BRACHET, J. 1955. *Biophys. Biochem. Acta.* **18**: 247.
14. HELLER, I. M. & M. J. KOPAC. 1955. *Exptl. Cell Research.* **8**: 62.
15. COHEN, A. I. 1957. *J. Biophys. Biochem. Cytol.* **3**: 923.
16. HOLTER, H. & E. ZEUTHEN. 1948. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **26**: 277.
17. PRESCOTT, D. M. 1955. *Exptl. Cell Research.* **8**: 256.
18. DALTON, A. J. 1955. *Anat. Record.* **121**: 281.
19. HOGNESS, D. S., M. COHEN & J. MONOD. 1955. *Biochem. Biophys. Acta.* **16**: 99.
20. PRESCOTT, D. M. 1956. *Exptl. Cell Rev.* **11**: 86.
21. JAMES, T. W. 1954. *Biochem. Biophys. Acta.* **15**: 367.
22. FRIEDRICH-FREKSA, H. & F. KAUDEWITZ. 1955. *Z. Electrochem.* **55**: 575.
23. HOGBOOM, G. W. & W. C. SCHNEIDER. 1952. *J. Biol. Chem.* **197**: 611.
24. MALKIN, A. & O. F. DENSTEDT. 1956. *Can. J. Biochem. Physiol.* **34**: 130.
25. BALTUS, E. 1954. *Biochem. Biophys. Acta.* **15**: 263.
26. BALTUS, E. 1956. *Arch. Intern. Physiol.* **64**: 124.
27. COHEN, A. I. 1956. *J. Biophys. Biochem. Cytol.* **2**: 15.
28. BRACHET, J. 1951. *Nature.* **180**: 205.
29. BURCH, H. B., C. A. STORVICH, R. L. BICKNELL, H. C. KUNG, L. G. ALEJO, W. A. EVERHART, O. H. LOWRY, C. G. KING & O. A. BESSLY. 1955. *J. Biol. Chem.*, **212**: 897.
30. ALIVISATOS, S. G. A., S. KASHKET & O. F. DENSTEDT. 1956. *Can. J. Biochem. Physiol.* **34**: 46.
31. KISSANE, J. & E. ROBINS. 1958. *J. Biol. Chem.* **233**: 184.
32. IVERSON, R. M. & A. C. GIESE. 1957. *Exptl. Cell Research.* **13**: 213.
33. SCHERBAUM, O. 1957. *Exptl. Cell Research.* **13**: 24.
34. GOLDSTEIN, L. & W. PLAUT. 1955. *Proc. Natl. Acad. Sci.* **41**: 874.
35. RABINOVITCH, M. & W. PLAUT. 1956. *Exptl. Cell Research.* **10**: 120.
36. BRACHET, J. 1950. *Experientia.* **6**: 294.
37. PRESCOTT, D. M. 1955. *Exptl. Cell Research.* **9**: 328.
38. ANDRESEN, N., C. CHAPMAN-ANDRESEN & H. HOLTER. 1952. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **28**: 189.
39. JAMES, T. W. & C. P. READ. 1957. *Exptl. Cell Research.* **13**: 510.

TRACER STUDIES IN AMOEBAE

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Radioactive isotopes have been used with the free-living amoebae for less than ten years. The amount of basic information, of insight into the workings of the cell that this work has produced attests both to the usefulness of this organism as a laboratory animal and to its willingness to endure considerable internal shattering for the sake of a comfortable glass-bowl existence. In my own experience as well as that of others (for example, Friedrich-Freksa and Kaudewitz, 1951) it has been virtually impossible to disturb the behavior of the cell by high doses of ingested isotope: cells assayed at several thousand counts per minute appear to metabolize and divide quite normally.

The isotope work in amoebae has been done with two species, *Amoeba proteus* and *Chaos chaos*, and falls into two broad experimental lines: general physiology and the function of the nucleus. This classification leaves out one very fascinating experiment (Friedrich-Freksa and Kaudewitz, 1953) which, albeit inconclusive, deserves some discussion. I shall return to this experiment later. The studies dealing with the general physiology of the cell have been done almost entirely by or in conjunction with the Carlsberg group (Andresen *et al.*, 1952; Andresen *et al.*, 1953; Chapman-Andresen and Holter, 1955; Chapman-Andresen and Prescott, 1956; Chapman-Andresen and Robinson, 1953), using *C. chaos* primarily. These investigators have studied the fate of ingested C^{14} in general and C^{14} -glucose in particular. In the course of this work they have devoted a great deal of effort to the development and calibration of both counting and autoradiographic methods for the detection and localization of the isotope in the cells. The work has led to a series of very beautiful investigations of pinocytosis. In view of the fact that these studies are being discussed in this monograph by some of the people most directly involved, I shall not presume to review the work here.

The remainder of the general physiological work consists of three studies which concerned themselves with the permeability of the amoeba's cell membrane. Pigón and Zeuthen (1951) established the usefulness of the Cartesian diver method for demonstrating the D_2O content of the cell. Prescott and Mazia (1954) used this method to examine possible differences in permeability to D_2O of whole amoebae, nucleated fragments, and enucleated fragments. They found none. As we shall see later, however, there are differences in uptake of more complex organic molecules between these three cell types. Whether these differences are due to simple membrane permeability differences, whether active uptake is involved, or whether differences in pinocytosis lead to these results are questions that are open at the present time. Brachet *et al.* (1957) have checked on the uptake of labeled ribonucleic acid by living amoebae. They assume that this large molecule enters by pinocytosis. Chapman-Andresen and Prescott (1956), however, noted that RNA as such was toxic to amoebae and did not lead to the formation of detectable pinocytosis channels. The fact

that Brachet and his co-workers did not obtain identical results with yeast RNA and tobacco mosaic virus RNA suggests that this problem requires further study.

The most fascinating and elusive problems in any biological system are those concerning the functioning of the system as a whole, the integrating mechanisms that connect the various biochemical reactions into a series of patterns characteristic of the system in different stages of development. The individual reactions as well as the control or integrating mechanisms are dynamic processes and therefore present experimental situations to which tracer methodology is ideally adapted. Before an integrated system can be understood as such it is obviously essential that the various component reactions be outlined and characterized. If this system is a cell rather than an amorphous one, we should like to go one step further and identify component reactions with component structures. An uninucleate amoeba such as *A. proteus* presents us with a system where a beginning can be made quite easily: nucleus and cytoplasm are readily separable without serious experimental difficulties. It is not surprising, therefore, that this quality of the amoeba has been combined with the advantages of tracers, that we have been able to learn a good deal about nuclear and cytoplasmic function and nuclear-cytoplasmic interaction from this experimental system.

The first published amoeba tracer experiment sought an answer to a question pertinent to this area of investigation. Mazia and Hirshfield (1950) compared the uptake of $P^{32}O_4$ from solution by enucleate amoeba fragments with that of nucleate fragments and whole cells. They found a striking difference between the uptake of enucleates and nucleates and concluded that the nucleus was very much involved in the cell's uptake of inorganic phosphate. The experimental results of Mazia and Hirshfield have been confirmed by Brachet (1952), who further noted that although appreciable amounts of P^{32} were incorporated into phospholipids, most of the isotope remained in the acid-soluble fraction. This fact permitted the interesting speculation that the nuclear influence is felt as an effect on phosphorylating systems within the cell. Mazia and Prescott (1954) utilized the demonstrable difference in $P^{32}O_4$ uptake between nucleates and enucleates to examine the uptake characteristics of the dividing cell as contrasted with those of the interphase cell. They found that with respect to $P^{32}O_4$ uptake the dividing cell resembles the enucleate rather than the nucleate interphase cell. While it is possible to use this evidence in support of the general concept that the cell's physiological activity is at a low during division, we might gain further insight by considering the probability that the dividing cell is a rather close counterpart to the enucleate cell in several respects. The dividing cell is a considerably more homogeneous system than the interphase cell: the nuclear membrane is absent or modified; the nucleoli have disappeared as structural entities; although the chromosomes are present, it is unlikely that they are exerting their genetic function at this stage. Insofar as any of the functions of the interphase cell depend upon a definite spatial organization of the cell's components, these functions should be absent or reduced when this organization is not present. The degree to which any cell function is depressed during division would thus be an indication of its dependence on the structural

organization characteristic of the interphase cell. It would be of some interest, in this connection, to screen the differences between nucleate and enucleate cells and study the most drastic of these in intact cells during division.

A more specific cellular activity, protein synthesis, has also been approached from the viewpoint of nuclear control with tracer experimentation in *A. proteus*. Mazia and Prescott (1955) have investigated the possible role of the nucleus in the incorporation of S^{35} -methionine into cellular proteins. They concluded that the nucleus is directly involved in at least a part of the synthesis of the cell's proteins. The same problem has also been studied by Ficq (1956) and Brachet and Ficq (1956) through the utilization of another labeled amino acid, C^{14} -phenylalanine. Both series of experiments were designed around the measurement of tracer incorporation into the acid-insoluble fraction in nucleate and enucleate amoebae as a function of time after enucleation. Both series led to essentially the same conclusions: amino-acid incorporation is reduced in the absence of the nucleus; the reduction is evident within a very short time after enucleation. Mazia and Prescott's data differ from those of Ficq in the extent of the reduction. At the present state of our knowledge this disagreement cannot be fully evaluated as to significance. Brachet (1957) has shown that not all proteins respond similarly to enucleation; neither methionine nor phenylalanine can be considered as quantitatively representative of the amino acids of all the amoeba's proteins; there is reason to believe that methionine is incorporated into nuclear proteins in amoeba to a greater extent than into cytoplasmic proteins at certain stages of the division cycle (Plaut, Prescott, and Mazia, unpublished observations); the same appears to be true for phenylalanine. In point of fact, Mazia and Prescott had suggested the nuclear synthesis of some cytoplasmic protein as a possible explanation of their data. In the absence of any evidence for the transfer of proteins from nucleus to cytoplasm—at a time other than division—this suggestion does not appear to constitute a very likely working hypothesis. The general drop in amino acid incorporation with time after enucleation is consistent with the hypothesis that the RNA level of a system is roughly indicative of its capacity for protein synthesis (Brachet, 1955). The data of Ficq and of Ficq and Brachet are approximately in agreement with what one would expect by noting the decrease in RNA in enucleate cells with time (Brachet and Ficq, 1956). This would mean that the more dramatic decrease in protein synthesis suggested by the Mazia and Prescott data could be explained only by assuming that the nucleus itself synthesizes and retains a considerable amount of methionine containing protein. This assumption is, of course, testable. One might suggest that the synthesis of some of the chromosomal protein is responsible for this methionine incorporation.

A fairly extensive series of tracer experiments has concerned itself with ribonucleic acid in *A. proteus*. All of these studies have been influenced by the important role that this macromolecular substance appears to play in the control of cellular synthetic processes. *A. proteus* contains large amounts of RNA in both its cytoplasm and its nucleus. In an experiment designed to corroborate the cytochemically demonstrated loss of nuclear RNA at the time of division with C^{14} -adenine-labeled RNA (Rabinovitch and Plaut, 1956) we found that the nuclear RNA label did not remain constant even in nondividing cells.

The time over which a decrease was noted was too short to make the results explicable in terms of total RNA decrease. Two possible explanations offered themselves: nuclear RNA "cooled" by exchange of unlabeled with labeled adenine; nuclear RNA was transferred to the cytoplasm between divisions while the nuclear membrane system remained intact. The latter possibility was of particular interest because of its bearing on: (1) the hypothesis that RNA served as transmitting agent of genetic specificity from nucleus to cytoplasm; and (2) the much-discussed hypothesis that nuclear RNA serves as a precursor for cytoplasmic RNA. It was possible to test the possibility of interphase transfer by transplanting nuclei whose RNA was labeled with P^{32} to unlabeled cells (Goldstein and Plaut, 1955). Under these experimental conditions it could be shown that RNA or P^{32} -containing RNA precursor did move from nucleus to cytoplasm and that, moreover, it was unlikely that a similar movement occurred in the opposite direction. This study thus supported the general contention that nuclear RNA was linked to the RNA located in the cytoplasm and showed that the intermediacy of RNA in nuclear gene control of cytoplasmic processes was mechanically possible and could operate across a nuclear membrane (the loss of nuclear RNA label and the appearance of labeled RNA in the cytoplasm took place before the cells had an opportunity to divide).

It was quite obvious at this time that the demonstration of a transfer of RNA from nucleus to cytoplasm told us very little about: (1) the relative contribution of this RNA to the total RNA in the cytoplasm; (2) the role played by this RNA in the cytoplasm; and (3) the generality of this phenomenon.

The first of these questions can be stated more directly: What does the cytoplasm contribute to the RNA found within it? Does it synthesize RNA? This question has proved itself amenable to experimental attack. A series of C^{14} -labeled precursors has been used in several laboratories on enucleate fragments of *A. proteus*; adenine (Skreb-Guilcher, 1956; Plaut and Rustad, 1956, 1957); uracil (Prescott, 1957; Plaut and Rustad, 1959); and orotic acid (Plaut and Rustad, 1959). Autoradiographic detection methods were used in all of these studies. With the exception of the data published by Prescott, there is general agreement: the enucleate cytoplasm is capable of incorporating these precursors into RNA. Prescott failed to obtain incorporation of uracil into the RNA of enucleate cells. In a more recent experiment, however, we have observed uracil incorporation under these conditions. This incorporation differs from that of adenine and orotic acid in two respects: there is less of it, and a significant fraction of the acid-insoluble label is not removable by ribonuclease. These circumstances suggest that uracil may not be a very dependable precursor for RNA studies in amoeba in our present state of ignorance of the nucleic acid metabolism in this organism.

Good quantitative data on the incorporation of precursors into the cytoplasmic RNA of nucleate and enucleate cells are not available at present. It is possible, however, to make some semiquantitative statements of a tentative nature. As a result of the adenine incorporation studies we had concluded (Plaut and Rustad, 1957) that although the average amount of labeled RNA in enucleate cells was below that of nucleate cytoplasm incubated for similar periods in the presence of the isotope, it did not necessarily follow that enu-

cleate cytoplasm incorporated the isotope at a lower rate. We offered two reasons for this conclusion: (1) both nucleate and enucleate cytoplasm showed a great deal of variability from cell to cell in labeling intensity; the most heavily labeled enucleates approximated the more lightly labeled nucleate cells; and (2) the fact that the average adenine uptake of enucleates is well below that of nucleates could be a sufficient explanation for the lower average incorporation of the enucleate cells. The more recent studies with C^{14} -orotic acid bring out this point even more forcefully. As with adenine, the average incorporation of enucleate cytoplasm is probably below that of nucleate cytoplasm. As with adenine, there is a great variability in labeling intensity in both nucleate and enucleate cells. However, enucleate cells with considerably more cytoplasmic RNA labeling than corresponding nucleate cells occurred with appreciable frequency. These data suggest, as a tentative conclusion, that the rate of orotic acid incorporation into the enucleate cytoplasm's RNA does not differ appreciably from that in nucleate cytoplasm. A cell-to-cell comparison of total orotic acid uptake in nucleates as well as enucleates is obviously needed to test this conclusion.

Can we speak of this incorporation as synthesis of RNA? With adenine, considerable caution is necessary. It has been suggested (Brown, 1956) that adenine as such may be incorporated into the RNA molecule by direct exchange. Orotic acid or, rather, the pyrimidines derived from this precursor, appears less likely to be subject to such direct incorporation. In short, in contrast to adenine, orotic acid incorporation into RNA is reasonably likely to indicate RNA synthesis. It should be pointed out here that the general validity of the assumption that RNA is subject to extensive constituent "turnover" is by no means clearly established. There are at least two sets of data, one in *A. proteus* (Friedrich-Freksa and Kaudewitz, 1951) and one in mouse tissue culture cells (Graham and Siminovitch, 1957) suggesting that RNA phosphorus is retained well during several generations.

We must ask a second question, however, about the RNA made in the cytoplasm in an enucleate cell. Is it the same RNA as is made or found in the cytoplasm of the nucleate cell? For the time being no ready answer can be given to this question. The problem may be approached experimentally by putting the previously mentioned incorporation data on a strict quantitative basis and checking the comparative rates, and by subjecting the RNA of the enucleate and nucleate cytoplasm to base ratio analysis.

If we assume that the RNA made by the enucleate cytoplasm is the same as that made in the cytoplasm of the nucleate cell (this assumption derives some slight support from a recent study of the invariability of bacterial RNA made under varying conditions by Lombard and Chargaff (1957), we have a tentative answer to one of the questions raised by the demonstration of RNA transfer from nucleus to cytoplasm: the direct contribution of the nucleus to cytoplasmic RNA is more likely to be small than large relative to total cytoplasmic RNA.

As regards the role played by the nuclear RNA in the cytoplasm, the amoeba experiments have provided us with no information beyond the general association of RNA with protein synthesis already mentioned. One might postulate

that, if there is only a small RNA contribution from nucleus to cytoplasm, then cytoplasmic RNA synthesis could be all or in part a replication of nuclear RNA. Incorporation experiments with enucleate cells incubated with labeled precursors at different times after enucleation may provide some clues in this connection. We are also in the dark as to which of the nuclear RNAs is involved in the transfer. For this particular question the amoeba may not be the ideal organism in view of the obscurity of the nucleolar picture. There is one recent suggestion (Osawa *et al.*, 1957) that in calf thymus cells it is the nonnucleolar RNA that may be moving from nucleus to cytoplasm.

The third basic question raised by the nuclear transplant experiment, that inquiring into the generality of nuclear RNA transfer, has been approached experimentally in *Acelabularia* (Stich and Plaut, 1958) since that organism provided the strongest evidence against the assumption that nuclear RNA is involved directly in cytoplasmic function. The experiment compared the behavior of nucleate and enucleate fragments of the alga after ribonuclease treatment. The results were consistent with the hypothesis that nuclear RNA, that is RNA coming from the nucleus, is essential for the normal functioning of the cytoplasm.

In summarizing these studies I am inclined to conclude that both the nucleus and the cytoplasm carry on RNA synthesis, that there is transfer of RNA from nucleus to cytoplasm across the nuclear membrane system, and that the amount transferred is relatively small if viewed from the side of the cytoplasm. It should be evident from the above discussion that this constitutes little more than a beginning in the examination of RNA as a possible intermediary agent between nucleus and cytoplasm. The chief value of the work done thus far lies, in the tentative frame-work for working hypotheses it provides and in the experimentally approachable questions it has raised.

I propose now to return to the one tracer experiment in *Amoeba*, which does not fit the categories of physiology or nuclear cytoplasmic interactions as used here. The experiment was designed to provide some information on the multiplicity of structural chromosomal subunits. Friedrich-Freksa and Kaudewitz (1953) labeled *A. proteus* cells with P^{32} , blocked division long enough to permit most of the P^{32} atoms to decay, and then correlated cell lethality with number of cell divisions. They found that after 35 divisions per cell a very large number of cells died. The assumptions underlying the interpretation of the data were these: P^{32} will be incorporated into the DNA of the amoeba's chromosomes, will decay there, and leave a basic structural strand defective. During replication the defective strand will result in a second defective strand. Division involves the random segregation of all the basic structural strands into two sets, the daughter chromosomes. When a whole chromosome consists of strands that are defective, the cell receiving this chromosome will die. The statistical evaluation of the data suggested that the amoeba's chromosomes consist of bundles of 16 basic strands. Although this estimate is in fair agreement with the current ideas of electron microscopists on chromosomes in general, it is unlikely that very many cytologists or biophysicists will accept the assumptions made in the analysis. In the present context I shall restrict myself to pointing out one serious difficulty: P^{32} labeling of DNA has not been demonstrated in *A. pro-*

teus thus far. Either this organism has no DNA or the amount of DNA present is very small. Staining suggests that the second explanation is the more probable one since the pictures obtained show a very low concentration of dye. This permits us to explain away our inability to label the DNA with P^{32} (as well as with adenine and orotic acid), but raises the question of whether the aforementioned lethality had anything to do with the amoeba's DNA.

During the last two months we have incubated *A. proteus* with high-specific-activity tritiated thymidine. Some of the cells, when analyzed autoradiographically, showed a low level of incorporation into the nucleus. However, there was also distinct cytoplasmic incorporation. Both the nuclear and cytoplasmic radioactivity could be removed by desoxyribonuclease. In view of the fact that the stock culture from which these cells were taken died out shortly afterward, it is possible that at least the cytoplasmic incorporation was attributable to an infective agent. The experiment is being repeated at the present time under conditions that preclude infection. It is our hope that the nuclear incorporation was not due to extraneous factors and that we shall be able to obtain some information on the amoeba's DNA.*

References

1. ANDRESEN, N., C. CHAPMAN-ANDRESEN & H. HOLTER. 1952. Autoradiographic studies on the amoeba *Chaos chaos* with ^{14}C . Compt. rend. trav. Lab. Carlsberg, Sér. chim. **28**: 189-220.
2. ANDRESEN, N., C. CHAPMAN-ANDRESEN, H. HOLTER & C. V. ROBINSON. 1953. Quantitative autoradiographic studies on the amoeba *Chaos chaos* with ^{14}C . Compt. rend. trav. Lab. Carlsberg, Sér. chim. **28**: 499-540.
3. BRACHET, J. 1952. The role of the nucleus and cytoplasm in synthesis and morphogenesis. Symposia Soc. Exptl. Biol. **6**: 173-200.
4. BRACHET, J. 1955. Action of ribonuclease and ribonucleic acid on living amoebae. Nature. **175**: 851-853.
5. BRACHET, J. 1957. Biochemical Cytology. Academic Press. New York, N. Y.
6. BRACHET, J., M. BRIERS & Y. THOMAS. 1957. The uptake of ribonuclease and ribonucleic acid by living amoebae. Biochem. J. **66**: 14.
7. BRACHET, J. & A. FICQ. 1956. Remarques à propos du rôle biologique des acides nucléiques. Arch. Biol. **67**: 431-446.
8. BROWN, G. B. 1956. Chemical pathways of nucleic acid biosynthesis. Federation Proc. **15**: 823-826.
9. CHAPMAN-ANDRESEN, C. & H. HOLTER. 1955. Studies on the ingestion of ^{14}C glucose by pinocytosis in the amoeba *Chaos chaos*. Exptl. Cell Research. Suppl. **3**: 52-63.
10. CHAPMAN-ANDRESEN, C. & D. M. PRESCOTT. 1956. Studies on pinocytosis in the amoebae *Chaos chaos* and *Amoeba proteus*. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **30**: 57-78.
11. CHAPMAN-ANDRESEN, C. & C. V. ROBINSON. 1953. The assay of ^{14}C labelled amoebae *in vivo*. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **28**: 343-357.
12. FICQ, A. 1956. Incorporation de phénylalanine- $3-^{14}C$ dans les fragments nucléés et anucléés d'amibes. Arch. Intern. Physiol. Biochim. **64**: 129-130.
13. FRIEDRICH-FREKSA, H. & F. KAUDEWITZ. 1951. Versuche mit P^{32} an *Amoeba proteus* and Hühnerpestvirus. Z. Elektrochem. **55**: 575-576.
14. FRIEDRICH-FREKSA, H. & F. KAUDEWITZ. 1953. Letzle Spatfolgen nach Einbau von P^{32} in *Amoeba proteus* und ihre Deutung durch genetische Untereinheiten. Z. Naturforsch. **8b**: 343-355.
15. GOLDSTEIN, L. & W. PLAUT. 1955. Direct evidence for nuclear synthesis of cytoplasmic ribose nucleic acid. Proc. Natl. Acad. Sci. **41**: 874-880.

* *Addendum*. Subsequent experiments suggest that both nuclear and cytoplasmic incorporation of thymidine occur in apparently healthy cells. (See Plaut, W. & L. A. Sagan. 1958. "Incorporation of thymidine in the cytoplasm of *Amoeba proteus*." *J. Biophys. Biochem. Cytol.* **4**: 843-846.)

16. GRAHAM, A. F. & L. SIMINOVITCH. 1957. Conservation of RNA and DNA phosphorus in strain L mouse cells. *Biochim. Biophys. Acta.* **26**: 427-428.
17. LOMBARD, A. & E. CHARGAFF. 1957. Aspects of the invariability of a bacterial ribonucleic acid. *Biochim. Biophys. Acta.* **25**: 549-554.
18. MAZIA, D. & H. I. HIRSHFIELD. 1950. The nucleus dependence of P^{32} uptake by the cell. *Science.* **112**: 297-299.
19. MAZIA, D. & D. M. PRESCOTT. 1954. Nuclear function and mitosis. *Science.* **120**: 120-122.
20. MAZIA, D. & D. M. PRESCOTT. 1955. The role of the nucleus in protein synthesis in amoeba. *Biochim. Biophys. Acta.* **17**: 23-34.
21. OSAWA, S., K. TAKATA & Y. HOTTA. 1957. Some aspects of the relation between nuclear and cytoplasmic ribonucleic acids. *Biochim. Biophys. Acta.* **25**: 656-657.
22. PIGÓN, A. & E. ZEUTHEN. 1951. Cartesian diver balance in permeability studies. *Experientia.* **7**: 455-456.
23. PLAUT, W. & R. C. RUSTAD. 1956. Uptake of adenine-8- ^{14}C by whole and fractional amoebae. *Nature.* **177**: 89-90.
24. PLAUT, W. & R. C. RUSTAD. 1957. Cytoplasmic incorporation of a ribonucleic acid precursor in *Amoeba proteus*. *J. Biophys. Biochem. Cytol.* **3**: 625-630.
25. PLAUT, W. & R. C. RUSTAD. 1959. The incorporation of uracil- C^{14} into RNA in the cytoplasm of *Amoeba proteus*. *Biochim. Biophys. Acta.* In press.
26. PRESCOTT, D. M. 1957. The nucleus and ribonucleic acid synthesis in *Amoeba*. *Exptl. Cell Research.* **12**: 196-198.
27. PRESCOTT, D. M. & D. MAZIA. 1954. The permeability of nucleated and enucleated fragments of *Amoeba proteus* to D_2O . *Exptl. Cell Research.* **6**: 117-126.
28. RABINOVITCH, M. & W. PLAUT. 1956. Cytochemical and autoradiographic observations on nuclear ribonucleic acid in *Amoeba proteus*. *Exptl. Cell Research.* **10**: 120-124.
29. SKREB-GUILCHER, Y. 1956. Cited by J. Brachet. 1957. *Biochemical Cytology*. Academic Press. New York, N. Y.
30. STICH, H. & W. PLAUT. 1958. The effect of ribonuclease on protein synthesis in nucleated and enucleated fragments of *Acetabularia*. *J. Biophys. Biochem. Cytol.* **4**: 119-121.

EFFECTS OF SELECTED CHEMICAL AGENTS ON AMOEBAE*

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For years biologists have concerned themselves with the physiological and biochemical activities of the amoeba. In elucidating the problems concerned with these activities one method of attack has been that of investigating the effects of chemical agents on the amoeba. Thus considerable data has been accumulated on the effects of many chemical substances on such important phenomena as the growth, respiration, and locomotion of the amoeba.

This review has as its main objective an analysis of the reactions of amoebae to certain selected chemical agents. The latter include adenosine triphosphate, which has been suggested as a possible source of metabolic energy for such activities as amoeboid form and locomotion. In this paper particular attention is given to experiments concerned with the effects of ATP and related compounds and the phosphorylative inhibitor, dinitro-o-cresol. Other chemical substances discussed are inorganic salts, anesthetics, inhibitors (of respiration and growth), proteins, and nucleic acids.

Methodology

Before discussing the influence of the various reagents on the amoeba, it may be well to review briefly some of the techniques employed in the application of these substances. The method of application is always an important consideration in analyzing the response of a cell to a chemical agent, since a substance may elicit different responses, depending upon the manner in which it is applied. Of the several procedures that have been used to test the effects of chemical substances on amoebae, four will be discussed.

Micrurgical procedures have contributed much to our knowledge of chemical effects on the cell, since they permit direct contact with localized areas and specific organelles within the cell. For example, the technique of microinjection allows a precise amount of the reagent to be injected directly into the chosen site at any desired rate. Furthermore, this method is useful for the introduction of otherwise impermeable substances into the animal. However, possible disturbances that may arise due to microinjection must be considered. Micrurgical procedures are not as suitable as immersion procedures for studies of the effects of chemical substances on the organism as a whole, since the action of a substance at one particular area of the cell may be quite different from its action on the entire organism. Micrurgy techniques have been used by several workers investigating the effects of ATP, proteins, and various physiological salt solutions.

The direct immersion of a specimen into the test solution is probably the most

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common procedure that has been employed. This method allows one to test the effect of a chemical on the entire rather than on a specific portion of the organism. However, with this technique, problems such as chemical solubility, modification of the culture media, and diffusion of the substance into the animal must all be considered in evaluating the responses of the animal. The direct immersion procedure has been employed in studies with numerous chemical agents, for example ATP, AMP, general inhibitors, and inorganic salts.

Another useful procedure is that of placing an animal in contact with the fumes of a chemical agent. Only volatile reagents can be used and their saturation in the culture media is a factor that must be considered. The advantage of this procedure is that it is possible to observe the specimen while it is being subjected to increasing concentrations of the test substance since, with increasing exposure to the fumes, greater amounts of the reagent saturate the culture medium. The saturation of the culture media continues until equilibrium between the reagent and the culture medium is attained. The use of this technique in the exposure of amoebae to chemical agents has been very effective in demonstrating the anesthetic effect of many reagents.

A unique method for studying the effects of chemical agents on the amoeba is that of incorporating the test substance into the animals' food. For example, one may place the food organism of the amoeba into a labeled substance and then permit the amoeba to feed on the radioactive organisms. This procedure has been used in studying RNA synthesis and digestion in amoebae.

Chemical Agents

Salts. No review of the action of chemical agents on the amoeba would be complete without a consideration of the effects of salts on amoeba protoplasm. The classic work, carried out about 30 years ago, has been to a large extent the basis for our present understanding of the physiology of amoeboid form and locomotion. From these comprehensive studies it is evident that salts not only affect form and locomotion in amoebae but also modify the viscosity and the internal hydrogen-ion concentration, as well as the surface characteristics of the cell.¹⁻³

Amoebae immersed into a solution of NaCl or KCl retract their pseudopodia, round up, and become quiescent, while the larger cytoplasmic granules aggregate and fall to the bottom of the cell. Similar results are obtained by injecting these same salt solutions directly into the cytoplasm, suggesting therefore that the cell is permeable to both Na and K ions. The plasmalemma becomes very fragile in NaCl solutions, but this does not occur in KCl solutions. On the other hand, CaCl₂ and MgCl₂ are far less toxic to the amoeba. A solution of a single salt (such as MgCl₂ or CaCl₂) may have different effects, depending upon the manner in which it is administered.^{4, 5} Amoebae move about quite actively when immersed in these solutions.⁴ However, injections of Mg⁺⁺ and, especially, of Ca⁺⁺ have a gelational effect on the cytoplasm. With injections of Ca⁺⁺, the amoeba reacts vigorously by pinching off the injured area. This may be due to the excessive production of acid that occurs as a result of calcium and magnesium injections.⁶ Both Chambers and Heilbrunn point out that calcium has an action antagonistic to both sodium and potassium.

The important role that calcium plays in amoeboid movement was illustrated by Pantin,⁷ who worked with marine amoebae. Pollack⁸ was able to show that the removal of calcium with calcium-precipitating agents causes inactivity and quiescence in *Amoeba proteus* and *Amoeba dubia*. He demonstrated that injections of calcium into these amoebae initiated active movement, but only for a short time. Mast⁹ has shown that the presence of salts facilitates attachment to the substrate, whereas nonelectrolytes do not aid this phenomenon.¹⁰ He observed that the hydrogen-ion concentration over a wide range (4.6 to 8.2) has very little influence on attachment. However, the optimal attachment in a balanced salt solution occurs between 6.6 and 7.0. Apparently calcium, magnesium, and potassium are additive for attachment, but not for locomotion. In a salt solution containing sodium and calcium there are two *pH* values, that is, 5.9 and 7.5, at which the rate of locomotion reaches a maximum.¹¹⁻¹⁴ However, in the presence of a single salt there is only one *pH* at which the rate of locomotion reaches a maximum. It is concluded from these studies that attachment is necessary for locomotion, but locomotion is largely dependent upon other factors, one of the most important being sol-gel transformations.⁹ In addition to their influence on attachment and locomotion, salts play an important role in ingestion of food by amoebae.¹⁵

The internal hydrogen-ion concentration of amoebae has been determined by immersion into, and injection of, various indicators. The values of the internal *pH* are between 6.9¹⁶⁻¹⁸ and 7.6.^{19, 20} These studies indicate that the cytoplasm has a remarkable buffer system and, although the *pH* of the intracellular inclusions may be shifted, the *pH* of the cytoplasmic matrix remains fairly constant. Buffer salts may be injected in relatively high concentrations without excessive harm; however, immersion in dilute buffer salts causes injury to the specimen.²¹

One of the most striking results of salts on the amoeba is their effect on the viscosity of the cell. This marked effect on amoeba viscosity has been shown by extensive centrifuge and micrurgy studies.^{5, 22-28} Heilbrunn and Daugherty found that calcium and magnesium decrease the viscosity, whereas sodium and potassium increase the viscosity of the plasmasol, as tested in *A. dubia*. Their studies of the plasmagel in *A. proteus* demonstrate that magnesium, potassium, and sodium decrease, whereas calcium increases the rigidity of the plasmagel structure. These results differ from those of Chambers and Reznikoff,⁴ who found that injections of calcium and magnesium increase the viscosity of the internal plasmasol. The extensive work of Kriszat²⁶⁻²⁸ indicates that in calcium-free solutions the viscosity of *Chaos chaos* decreases.

High salt concentrations also have an unusual effect on amoebae in that they cause pinocytosis. Mast and Doyle²⁹ and more recently Chapman-Andresen and Prescott³⁰ have reported that 20 to 50 per cent sea water solutions cause pinocytosis in *A. proteus* (the reader is referred to the papers of Holter and Marshall in this publication).

Anesthetics. Investigations of the action of anesthetics have been of considerable aid in elucidating problems concerning cell structure and function. Many volatile, fat-soluble substances have an anesthetic effect on amoeba. These agents frequently affect activities concerned with form and mobility.

Specific responses of the amoeba are used to measure the anesthetic effect. For example, insensitivity to the prodding of a microneedle,³¹ inability of contractile vacuole activity, incoordination of movement,³² and lack of response to bright illumination³³ are some of the criteria that have been utilized in studying the effects of anesthesia.

Experimentation with homologous substances such as alcohols^{32, 34} and paraffin oils³³ on amoebae indicates that, as the length of the carbon chain increases, there is a corresponding increase in the effect of the anesthetic. Hiller³⁵ attributes the narcotic action of alcohols to a decrease in surface tension that accompanies the modification of protoplasmic viscosity. Marsland³³ found that homologous series of paraffin oils are effective in producing narcosis. The short chained members produced narcosis when the amoeba were immersed in them; however, the long chain compounds (dodacane and tetradecane) did not seem to elicit a response since they were apparently impermeable to the cell surface. However, if they were injected into the amoeba, a response comparable to that of the short chain substances was recorded. Daugherty³⁴ reported a solution of the plasmagel in *A. proteus* with the application of high sublethal concentrations of alcohol. However, in *A. dubia* similar concentrations of the lower alcohols (methyl, ethyl, and propyl) produced an increase in plasmasol viscosity, whereas the higher alcohols (butyl and amyl) produced a decrease in plasmagel viscosity. When the concentrations of the higher alcohols are lowered, the plasmasol viscosity increases. Kitching³⁶ reported that high pressure represses the formation of pseudopodia and the clear blisters promoted by dilute ethanol solutions.

Recently Goldacre³¹ conducted a comprehensive study of anesthetics on *Amoeba discoides*. He found that anesthetics acted in a concentration that was roughly one-tenth saturation. In general, the response to the various anesthetics was similar. That is, an increase in the area of the cell membrane occurred that was caused by its lifting from the cytoplasm and moving outward. The action was reversible except for prolonged exposures and high concentrations of anesthesia. Prior to the time when the anesthetic became effective, the amoebae were hypersensitive and slight contact with the microneedle caused a violent contraction. Goldacre postulates from these experiments and others that the response of amoebae to touch may involve a type of enzymatic action of the membrane on the granular cytoplasm.

Inhibitors. Various agents have been tested with regard to their effects on respiration and growth in amoebae. It is evident from these studies that frequently the stimulatory or inhibitory effect of the agent is dependent on the concentration of the substance employed. Pace and his co-workers³⁷⁻⁴⁰ have shown that high concentrations of metabolic inhibitors (KCN, arsenite, ethyl urethane, and ethyl alcohol) inhibit both growth and respiration. On the other hand, sublethal concentrations of KCN and ethyl alcohol increases both growth and respiration. Pace³⁸ concludes from his studies that the cytochrome-cytochrome oxidase system is the mechanism chiefly involved in the oxidation of carbohydrate in *Pelomyxa*. Reich,⁴¹ studying respiration in soil amoebae (*Mayorella palestinesis*), found a similar stimulatory effect with azide and KCN. Susca and Wilber⁴² report that lethal concentrations of cyanide pro-

duce changes in size and shape of the nuclei as well as in the cytoplasmic granules.

The presence of oxygen is not essential for locomotion since it has been shown that locomotion of fresh water amoebae^{43, 44} as well as marine amoebae^{45, 46} can continue in the absence of oxygen for as long as 6 to 12 hours.

Such agents as DDT,^{47, 48} Tweens,⁴⁹ and nitrogen mustard^{50, 51} have been investigated on amoeba protoplasm. Ord and Danielli were able to study the nuclear-cytoplasmic interrelationships in amoebae by employing lethal and sublethal concentrations of nitrogen mustard. They found that this drug may cause extensive damage to the cytoplasm, and that the damage is not the result of nuclear injury. It was demonstrated by nuclear transplantation experiments that an amoeba with lethally damaged cytoplasm does not divide; on the other hand, if it has a normal cytoplasm and a damaged nucleus it is likely to divide several times before dying.

Proteins and nucleic acids. Since the early work of Schaeffer⁵² in 1917 on the reactions of amoebae to proteins, various experiments have been undertaken on the action of proteins and protein synthesis in amoebae. Marshak⁵³ studied changes in the hydrogen-ion concentration in amoebae following injections of egg albumin. Recently Kassel and Kopac^{54, 55} reported the effects of several proteins and nucleotides that were injected into amoebae. Chapman-Andresen and Prescott⁵⁰ found that numerous proteins and nucleic acids caused pinocytosis in nucleate and enucleate amoebae. The ingestion of labeled glucose and protein solutions in amoebae by pinocytosis has been investigated.^{56, 57} Further studies on digestion and metabolism in amoebae were carried out by feeding amoebae radioactive food and studying the incorporation of the labeled substances.⁵⁸⁻⁶⁰ Goldstein and Plaut⁶¹ were able to study RNA synthesis by feeding labeled food to amoebae and then transplanting the radioactive nuclei to enucleate hosts.^{62, 63}

Brachet⁶⁴⁻⁶⁶ in a study of protein synthesis reported the effects of RNA and RNAase on amoebae. He found that RNAase inhibits locomotion, whereas RNA and oxidized RNAase have little if any effect. By immersion of amoebae into solutions of RNA plus RNAase, he demonstrated that RNA protects the cell from the inhibitory effects of RNAase. Cytological investigations indicate a loss of basophilia in the cytoplasm and the nucleoli as a result of RNAase treatment. In addition, the studies show that RNA has a distinct stimulatory action with regard to cell multiplication in contrast to the inhibitory action of RNAase. In conjunction with radioactive incorporation studies, Brachet concluded that the incorporation of amino acids into proteins and the ability to reproduce are intimately linked to the RNA content of the cell.

Adenosine triphosphate. Extensive research on the energy requirements of protoplasmic sol-gel reactions has lead to the hypothesis that ATP may be the energy source in amoeboid form and movement. In 1942, Marsland and Brown⁶⁷ reported that the ATP system appears to be active in the gelation reactions of freshly prepared myosin sol. Recently, the importance of the ATP system in sol-gel transformations has received strong support from the work of Ts'o and his associates,^{68, 69} who have isolated and characterized an ATP-sensitive protein (myxomyosin) from an amoeboid slime mold (*Physarum poly-*

cephalum). The work of these investigators suggests that an ATP-sensitive protein may be involved in the sol-gel transformations that are observed in the intact plasmodia. Further evidence for the important role of ATP comes from the work of H. H. Weber and his colleagues,^{70, 71} who found that ATP initiates a variety of cellular movements in glycerol water-extracted cell models. Among the activities observed are anaphase movements of chromosomes, cellular furrowing, and contraction of fibroblasts.

The extensive work of Kriszat^{26, 28, 72, 73} on amoebae clearly indicates that ATP increases the cytoplasmic viscosity. Goldacre³¹ and Goldacre and Lorch⁷⁴ have reported that microinjection of ATP into *A. discoides* results in a solation of the plasmagel in the region of the injection, followed by a period of accelerated streaming. Goldacre³¹ attributes this phenomenon to a contraction and a subsequent liquefaction of the cortical gel; he compares the contraction and subsequent syneresis to the action of ATP on muscle.

Recently we⁷⁵ have reported the effects of ATP and related compounds on the form and movement in *A. proteus*. Amoebae exposed to high concentrations of ATP (0.002 M) exhibited an immediate and drastic alteration in form and activity. After an exposure of 20 to 30 min. the characteristic picture is one showing rounded, quiescent amoebae with small hyaline blebs and large central vacuoles. This reaction is similar to the one described by Kriszat⁷² for *C. chaos* when exposed to concentrations of 0.002 to 0.006 M ATP. Kriszat found that death occurred within 24 hours in amoebae exposed to concentrations greater than 0.005 M ATP. Similarly, Brachet⁷⁶ reported toxicity with high ATP concentrations. We found that the contraction and subsequent vacuolization with high ATP concentrations were completely reversible if the ATP-treated amoebae were thoroughly washed and transferred to fresh Brandwein solution; the amoebae recovered their normal form and movement within 2 to 3 hours. At 0.001 M ATP, it was observed that about 20 per cent of the amoebae exhibited rounded quiescence. Lower concentrations of ATP, namely 0.0005 M, had only a very slight effect on the amoebae and 95 per cent of the specimens exhibited normal form and movement for many days.

The extensive work of Marsland and his associates⁷⁷⁻⁷⁹ has shown that the pseudopodia of amoebae are unstable when subjected to hydrostatic pressure. When pressure is applied to amoebae the pseudopodia collapse and the amoebae round up into motionless spheres. The pressure necessary to cause this rounding is a function of the temperature, that is, the pressure level necessary to induce this condition becomes less as the temperature is lowered. In addition, it has been demonstrated that pseudopodial stability is related to the relative plasmagel strength (FIGURE 1). Thus, temperature and pressure represent a combination of variables, which provide a useful method for studying the action of various chemical agents on sol-gel equilibria in amoebae and for analyzing the relationships between gelation and physiological activity (see J. V. Landau elsewhere in these pages for a more comprehensive study of pressure and temperature on amoebae).

Experiments were conducted in which *A. proteus* were immersed for 30 min. in 0.0005 M ATP and then subjected to hydrostatic pressures at different temperatures. The minimum pressure necessary to cause 75 per cent of the ATP-

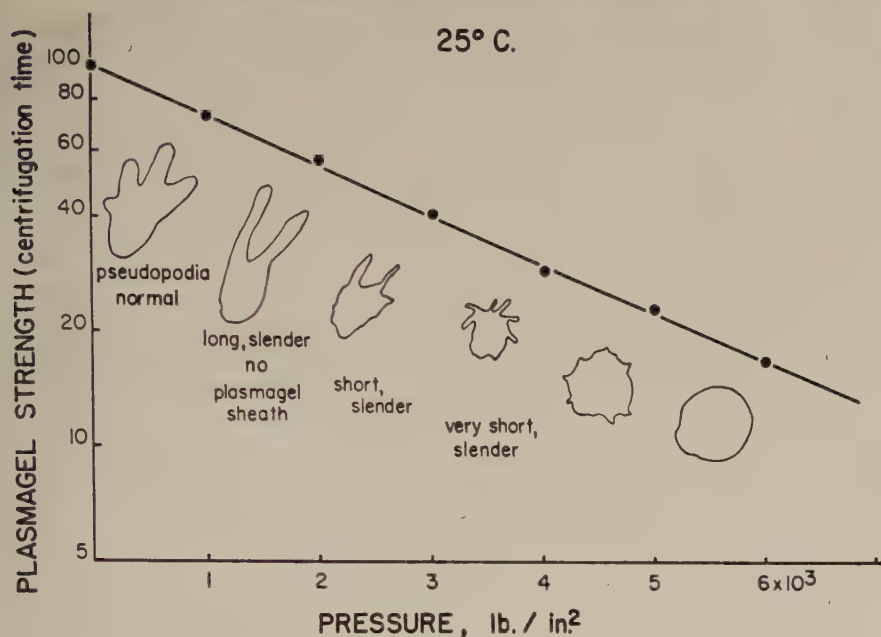


FIGURE 1. *Amoeba proteus*: plasmagel strength and amoeboid form in relation to high hydrostatic pressure. Data from Brown and Marsland⁷⁸.

treated specimens to become spherical, within 20 min. at a given temperature, was compared to the pressure level of nontreated controls. The pressure levels of ATP-treated specimens were appreciably greater than those of the corresponding controls at each of the temperatures studied. There was a 500-lb./in.² differential between the controls and the ATP-treated amoebae. At 10°, 15°, 20°, and 25° C it required 3500, 4500, 5500, and 6000 lb./in.² respectively, to induce a total loss of pseudopodia in 75 per cent of the ATP-treated specimens, whereas it required only 3000, 4000, 5000, and 5500 lb./in.² respectively, to round up the control amoebae (FIGURE 2). From the previous studies on amoebae these results would indicate⁷⁹ that the plasmagel system of the ATP-treated amoebae maintains a firmer plasmagel structure than nontreated specimens. The centrifugal and micrurgical studies of Kriszat^{26, 72} tend to support this view since he observed an increase in viscosity with regard to both the heavy and light cellular components in ATP-treated *C. chaos*.

The importance of ATP as an energy source in muscle,⁷⁰ suggested that this metabolite might contribute energy to the sol-gel cycle in cells generally. Numerous studies have been conducted on dividing cells in which the evidence indicates that ATP plays a predominant role. Studies on marine eggs have shown that 0.0005 M ATP increases the cortical plasmagel, a structure related to the plasmagel in amoebae.⁸⁰ Kriszat and Runnstrom⁸¹ have found that sea urchin eggs treated with ATP are more resistant to hypotonic cytolysis, a phenomenon that seems to be related to increased viscosity of the cytoplasm.

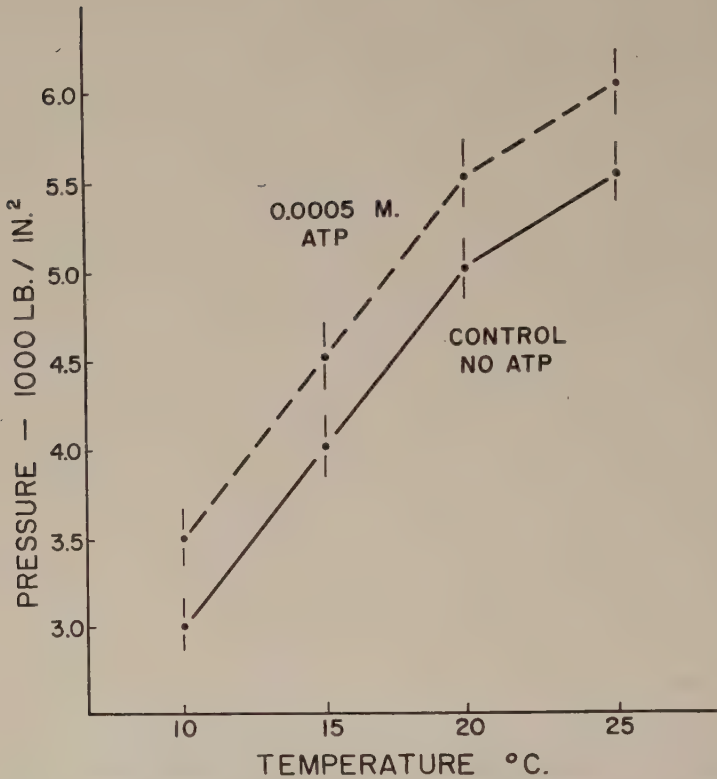


FIGURE 2. *Amoeba proteus*: the effect of 0.0005 M adenosine triphosphate on pseudopodia stability. The minimum pressure required to abolish pseudopodia is plotted as a function of temperature. Reproduced by permission from Zimmerman *et al.*⁷⁶

Theoretically, it is difficult to understand how a large molecule such as ATP can enter the amoeba by diffusion alone, but it cannot be denied that ATP has a decided effect on pseudopodial stability and on the internal viscosity of the cytoplasm. Kriszat²⁸ was able to show by appropriate staining techniques that ATP-treated amoebae and nontreated specimens stain differently when subjected to the acid dye aurantia. In the case of amoebae we could postulate that the ATP could be carried into the interior of the cell by continuous infolding of the posterior parts of the cell membrane, which Goldacre and Lorch⁷⁴ have shown to be responsible for the absorption of neutral red. Goldacre³¹ estimates that the membrane of an actively moving amoeba is renewed completely every 2 min., which would suggest that ATP could enter the cell quite readily if it were adsorbed on the cell surface. From the work of Mast and Doyle²⁹ and the recent studies of Chapman-Andresen and her associates,^{30, 57} it seems very possible that large molecular substances can enter the cell in still another manner, that is, by pinocytosis. It is also possible, however, that the ATP does not elicit its response by entering the cell as a large molecular unit but,

following the suggestions of Lindberg⁸² and Runnstrom and Kriszat,⁸³ the ATP merely transfers energy and/or labile phosphate across the cell surface to the subjacent ectoplasm.

Adenosine monophosphate and related compounds. It is important to ascertain to what extent, if any, the high-energy phosphate component of ATP is essential with regard to the pseudopodial stabilizing effect of ATP. ATP-related compounds such as adenosine monophosphate (AMP), adenosine (Ad), inorganic phosphate (Na_2HPO_4), and pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$) were studied on *A. proteus*. In this series of experiments, both the temperature (20°C) and pressure (5000 lb./in.^2) were kept constant. The criterion used in these experiments was the percentage of amoebae that retained some evidence of pseudopodia under the specified conditions of temperature-pressure.

High concentrations of AMP (0.008 M) caused a retraction of pseudopodia, blebbing, and vacuolization. This retraction was similar to that reported earlier using 0.002 M ATP. Lower concentrations of AMP gave progressively weaker effects. At a concentration of 0.006 M about 50 per cent and at 0.004 to 0.002 M about 10 per cent of the specimens exhibited retracted pseudopodia, blebbing, and vacuolization. Specimens immersed for 40 min. in 0.002 M AMP and subjected to a pressure of 5000 lb./in.^2 showed increased pseudopodial stability comparable to that of amoebae treated with 0.0005 M ATP. At a concentration of 0.0005 M AMP, the pressure studies indicate no increased pseudopodial stability as compared to the nontreated controls. It is evident, therefore, that the action of AMP is similar to that of ATP if the AMP concentration is four times that of the effective ATP concentration (FIGURE 3).

Adenosine in concentrations as high as 0.016 M and dibasic phosphate (Na_2HPO_4) in concentrations up to 0.002 M did not produce any appreciable effect on form and locomotion, and neither one had any stabilizing effect on the pseudopodia. In fact, adenosine seemed to have an adverse effect and made the pseudopodia distinctly less stable. Sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$) at a concentration of 0.001 M was toxic to the cell and produced considerable cytolysis. However, at 0.0001 M there was no improvement with regard to the ability of the amoebae to withstand the solating action of the pressure (FIGURE 3). Kriszat⁷² found that pyrophosphate and adenosine produced a temporary lowering in the viscosity, whereas Na_2HPO_4 and AMP initiated a temporary rise in the cytoplasmic viscosity. Kriszat reported that $\text{Na}_4\text{P}_2\text{O}_7$ and AMP were harmful to the animals and that, when immersed in these solutions, they could not survive longer than 24 hours.

In general, therefore, the work on amoebae and marine eggs⁸⁰ tends to indicate that adenosine triphosphate and adenosine monophosphate (in concentrations four times that of the effective ATP concentration) enhance the rigidity of the plasmagel structure.

Dinitro-o-cresol. This reagent was tested on amoebae, since it has been shown by the work of Loomis and Lipman⁸⁴ that dinitrophenol (a compound closely related to dinitro-o-cresol) prevents the orderly formation of energy-rich phosphate compounds during oxidation in cell-free homogenates. The reviews of Simon⁸⁵ and Clowes⁸⁶ indicate that dinitrophenol, including the methyl de-

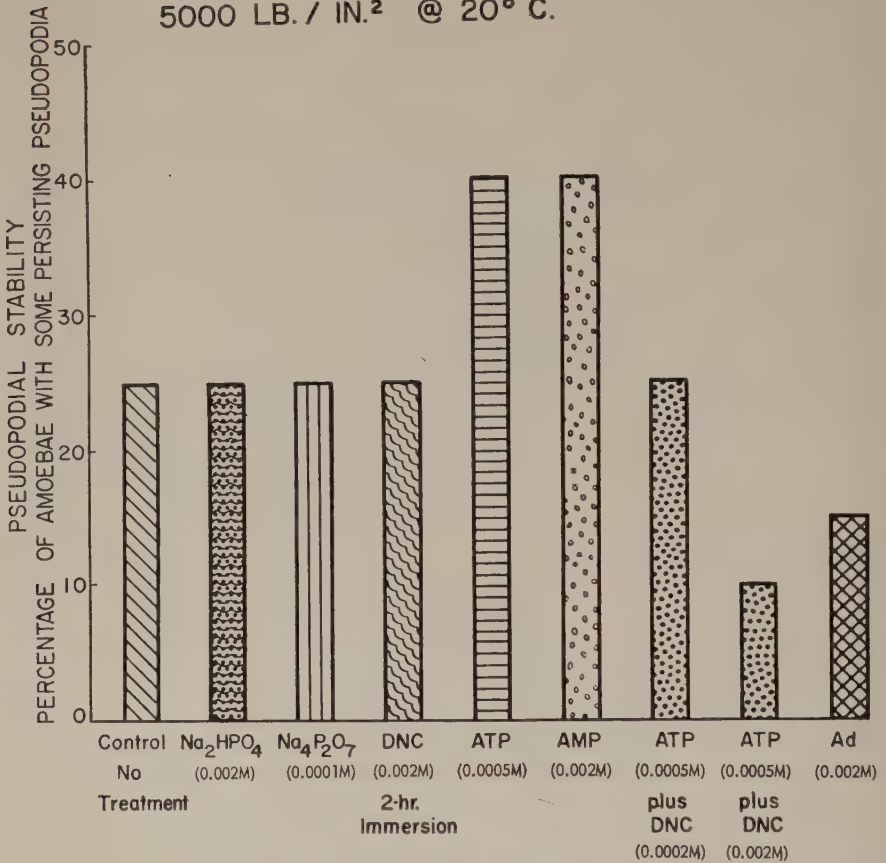
5000 LB. / IN.² @ 20° C.

FIGURE 3. *Amoeba proteus*: the effects of adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine (Ad), inorganic phosphates, dinitro-o-cresol (DNC), and combinations of ATP and DNC on pseudopodial stability. In each experiment the value illustrates the percentage of amoebae with definitely persistent pseudopodia after a compression of 30 min. at 5000 lb./in.² (20° C.). Reproduced by permission from Zimmerman *et al.*⁷⁵

rivative dinitro-o-cresol, interferes with the uptake of inorganic phosphate and the production of energy-rich phosphate. Therefore, 4,6 dinitro-o-cresol (DNC) was studied on amoeboid form and locomotion in order to ascertain what effect, if any, this reagent had on pseudopodial stability.

Amoebae immersed in concentrations of 0.002 M DNC for a period of 2 hours exhibited normal form and movement. However, at the end of 6 hours the specimens were rounded and inert and many were cytolized. In lower concentrations of DNC (0.001 to 0.0001 M) only a slight effect was visible after 6 hours. After 24 hours 95 per cent of the specimens in 0.001 M were quiescent and rounded, whereas only 10 to 20 per cent of the specimens in 0.0001 M exhibited inactivity.

Amoebae immersed in 0.002 M DNC for 2 hours and then exposed to pres-

sure (5000 lb./in.² at 20° C.) showed normal pseudopodial stability as compared with nontreated controls (FIGURE 3). It was not possible in these experiments to extend the immersion time beyond 2 hours since it was observed that an increasing number of amoebae began to retract their pseudopodia. In essence, therefore, the experiments with DNC indicate that this substance has little, if any, weakening effect on the structural state of the cortical cytoplasm of amoebae.

Ts'o and his co-workers⁶⁸ have reported that 0.001 M dinitrophenol stops protoplasmic streaming in myxomycete plasmodia within 5 min. and that 0.0002 M solutions retard streaming and finally stop it completely in 30 min. This is in contrast to the methyl derivative DNC which, at the same concentrations and for similar exposures, has little if any effect on protoplasmic streaming in amoebae.

Combinations (ATP and AMP) with dinitro-o-cresol. Although dinitro-o-cresol does not have any effect on form and locomotion in amoebae, except in high concentrations and over long exposures, it was thought worthwhile to study the combined effect of ATP plus DNC in order to establish whether the ATP effect could be blocked if appropriate concentrations of DNC were avail-

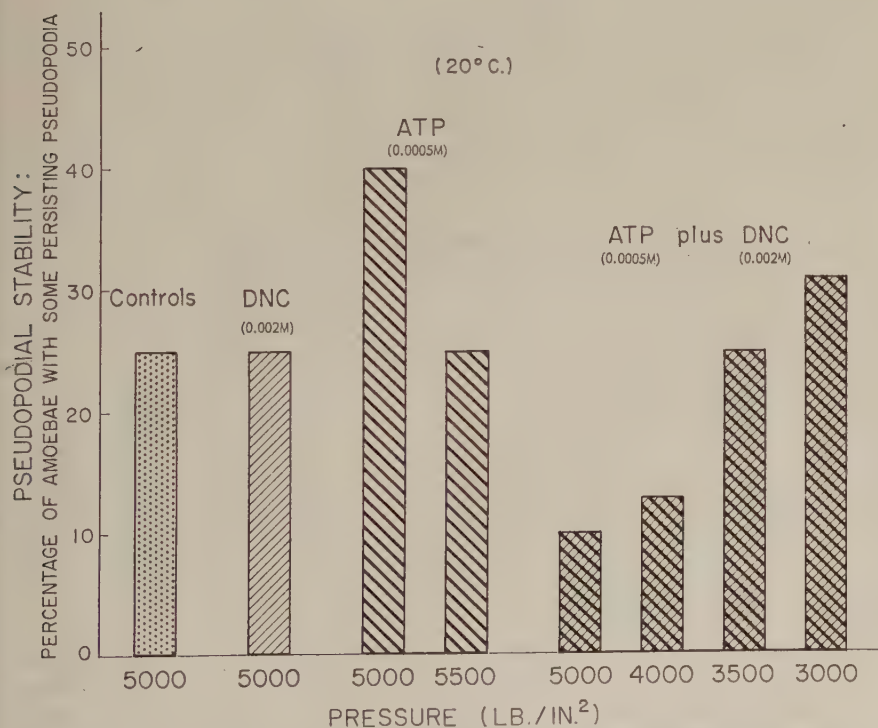


FIGURE 4. *Amoeba proteus*: combined action of adenosine triphosphate (ATP) and dinitro-o-cresol (DNC) upon pseudopodial stability in *A. proteus* in comparison with the action of each compound used singly. In each experiment the percentage of specimens with definitely persistent pseudopodia was determined after a standard compression period of 20 min.

able. Experiments were conducted to test pseudopodial stability after treatment with combinations of ATP plus DNC and AMP plus DNC. It was shown earlier in this paper that DNC alone does not modify pseudopodial stability as tested by pressure experiments. However, when 0.0005 M ATP was combined with 0.002 M DNC, the dinitro-o-cresol counteracted the adenosine triphosphate effect and there was a marked instability of the pseudopodia as tested by pressure. The results of these experiments are summarized in FIGURE 4. It may be seen from FIGURE 4 that there is a 1500 lb./in.² differential between the nontreated controls and the ATP-DNC-treated amoebae and that a 2000 lb./in.² difference is recorded if the ATP-DNC specimens are compared with the ATP-treated amoebae.

Further studies were conducted in order to establish the minimal concentra-

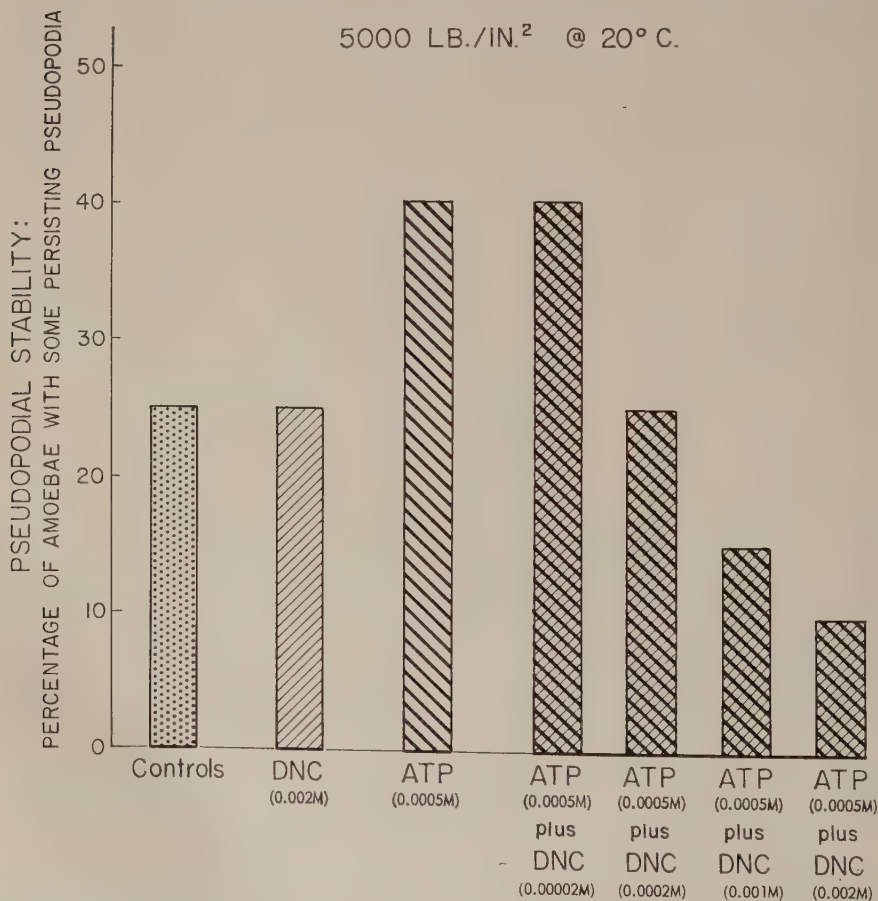


FIGURE 5. *Amoeba proteus*: the effects of varying the concentrations of dinitro-o-cresol (DNC) in combination with a standard concentration of 0.0005 M adenosine triphosphate (ATP) on pseudopodial stability. In each experiment not less than 100 specimens were used; each value illustrates the percentage of nonrounded specimens with some persistent pseudopodia after a 20-min. exposure to a pressure of 5000 lb./in.² at 20° C.

tion of DNC which, when combined with ATP (0.0005 M), would elicit an effect on pseudopodial stability. It was found that, as the concentration of DNC (in combination with ATP) was decreased, the pseudopodial stability was increased. At a concentration of 0.0002 M DNC, the stabilizing influence of ATP was exactly canceled and, at 0.00002 M DNC, the stabilizing influence of ATP was evident (FIGURE 5).

Since it was shown earlier that AMP in a concentration 4 times that of ATP tends to duplicate the ATP effects, AMP was substituted for ATP in the combination experiments. In concentrations up to 0.002 M AMP in combination with 0.002 M DNC, there was no appreciable effect on amoeboid form or pseudopodial stability.

General Discussion and Summary

The comprehensive studies on salts demonstrate conclusively that these agents are capable of modifying the structural as well as the physiological activities of the amoebae. The effects observed include alterations of shape, mobility, viscosity, and internal hydrogen-ion concentration. The experiments on anesthesia illustrate that the sensitivity of amoebae to a homologous series of substances, such as alcohols or paraffin oils, increases as the length of the carbon chain increases. Investigations on respiratory inhibitors reveal that they not only inhibit respiration and growth, but also may cause structural changes in the cell. Furthermore, inhibitors may also act in a stimulatory capacity depending, of course, on the concentrations employed. The comprehensive studies on proteins and nucleic acids shed new light on various problems of synthesis as well as nuclear-cytoplasmic interrelationships.

The investigations of adenosine triphosphate tend to suggest that this agent may be the energy source for sol-gel transformations in amoebae. Extensive evidence indicates that energy expended in cellular activities, such as amoeboid movement, is derived from the inherent contractility of the cortical plasmagel structure.^{75, 79, 80, 87} All available data suggest that the formation of a gel structure in protoplasm involves the bonding of certain protein components into a three-dimensional network and that the transition from sol to gel is an endothermic process involving a positive volume change. It is postulated, therefore, that the gelation reaction received energy from the hydrolysis of adenosine triphosphate.

A discussion of the formation of protein networks and the interaction of ATP has been given in an earlier publication;⁷⁵ however, a few points will be reviewed. We have postulated that the contraction that occurs prior to the dissociation of the protein network is the result of a forceful folding or shifting of the protein micelle into a compact form. It is proposed that the ATP initiates the contraction of the gel network when brought into proper spatial relationship with the cytoplasm. The contraction may involve an enzyme-substrate complex between the contractile protein and the adenosine triphosphate. Following contraction and dissociation of the protein network, the energy for re-formation of the gel structure would be derived from the ATP system.

The negative results obtained with the inorganic phosphates and adenosine indicate that these substances do not provide energy for the metabolic system.

On the other hand, the positive results with adenosine monophosphate suggest that the cell can use this compound, although not efficiently as ATP. Another possibility is that perhaps AMP provides a precursor for the build-up of ATP.

It is difficult, at this time, to interpret the action of the combined effects of adenosine triphosphate and dinitro-o-cresol, since ATP alone increases pseudopodial stability, whereas DNC alone has no appreciable effect. The combination experiments suggest that DNC cannot penetrate the membrane of the amoeba without the expenditure of energy. Perhaps the energy derived from adenosine triphosphate facilitates the transfer of dinitro-o-cresol across the cell surface and, once inside the cell, the DNC could interfere with the phosphorylation reactions within the cell. In this connection, it is interesting to note that amoebae treated simultaneously with ATP and parachloromercuribenzoic acid showed an irreversible contraction that was accompanied by a cessation of pseudopodial formation, whereas treatment with ATP or parachloromercuribenzoic acid alone did not affect the amoebae in the same fashion.⁷³

This report has summarized our present knowledge concerning the effects of chemical agents on amoebae. Contradictory reports are due in large part to differences in the techniques employed and in the criteria used to measure the responses. Hence methodology must be carefully considered in analyzing the effects of chemical agents. In conclusion, it should be emphasized that studies on the effects of chemical agents on amoebae have advanced our knowledge and understanding of the physiological and biochemical activities of the cell.

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References

1. PROSSER, C. L. 1950. Comparative Animal Physiology. : 630-639. Saunders. Philadelphia, Pa.
2. HEILBRUNN, L. V. 1952. An Outline of General Physiology. : 367-380. Saunders. Philadelphia, Pa.
3. CHAMBERS, R. & E. L. CHAMBERS. 1953. *Arzneimittel Forsch.* **3**: 322-325.
4. CHAMBERS, R. & P. REZNIKOFF. 1926. *J. Gen. Physiol.* **8**: 369-401.
5. HEILBRUNN, L. V. & K. DAUGHERTY. 1931. *Physiol. Zool.* **4**: 635-651.
6. REZNIKOFF, P. & H. POLLACK. 1928. *Biol. Bull.* **55**: 377-382.
7. PANTIN, C. F. A. 1926. *Brit. J. Exptl. Biol.* **3**: 275-295.
8. POLLACK, H. 1928. *J. Gen. Physiol.* **11**: 539-545.
9. MAST, S. O. 1929. *Protoplasma.* **8**: 344-377.
10. EDWARDS, J. G. 1924. *Brit. J. Exptl. Biol.* **1**: 571-595.
11. PITTS, R. F. & S. O. MAST. 1934. *J. Cellular Comp. Physiol.* **4**: 237-256.
12. PITTS, R. F. & S. O. MAST. 1934. *J. Cellular Comp. Physiol.* **4**: 435-455.
13. MAST, S. O. & C. L. PROSSER. 1932. *J. Cellular Comp. Physiol.* **1**: 333-354.
14. HOPKINS, D. L. 1928. *J. Morphol. Physiol.* **45**: 97-119.
15. MAST, S. O. & R. H. FENNELL. 1938. *Physiol. Zool.* **11**: 1-18.
16. CHAMBERS, R. 1928. *Biol. Bull.* **55**: 369-376.
17. POLLACK, H. 1928. *Biol. Bull.* **55**: 383-385.
18. CHAMBERS, R., H. POLLACK & S. HILLER. 1927. *Proc. Soc. Exptl. Biol. Med.* **24**: 760-761.
19. NEEDHAM, J. & D. M. NEEDHAM. 1926. *Proc. Roy. Soc. London.* **B99**: 383-397.
20. PANTIN, C. F. A. 1923. *J. Marine Biol. Assoc.* **13**: 24-69.

21. CHAMBERS, R. & P. REZNIKOFF. 1927. *J. Gen. Physiol.* **10**: 731-738.
22. HEILBRUNN, L. V. & K. DAUGHERTY. 1932. *Physiol. Zool.* **5**: 254-274.
23. HEILBRUNN, L. V. & K. DAUGHERTY. 1934. *J. Cellular Comp. Physiol.* **5**: 207-218.
24. THORNTON, F. E. 1935. *Physiol. Zool.* **8**: 246-254.
25. ANGERER, C. A. 1942. *Physiol. Zool.* **15**: 436-442.
26. KRISZAT, G. 1950. *Arkiv Zool.* **2**: 477-490.
27. KRISZAT, G. 1952. *Arkiv Zool.* **3**: 107-114.
28. KRISZAT, G. 1952. *Arkiv Zool.* **3**: 115-122.
29. MAST, S. O. & W. L. DOYLE. 1934. *Protoplasma.* **20**: 555-560.
30. CHAPMAN-ANDRESEN, C. & D. M. PRESCOTT. 1956. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **30**(5): 57-78.
31. GOLDACRE, R. J. 1952. *Symposia Soc. Exptl. Biol.* **6**: 128-144.
32. PANTIN, C. F. A. 1930. *Proc. Roy. Soc. London.* **B105**: 565-579.
33. MARSLAND, D. A. 1933. *J. Cellular Comp. Physiol.* **4**: 9-33.
34. DAUGHERTY, K. 1937. *Physiol. Zool.* **10**: 473-483.
35. HILLER, S. 1949. *Exptl. Cell Research. Suppl.* **1**: 374-377.
36. KITCHING, J. A. 1954. *J. Protozool.* **1** Suppl. : 13.
37. PACE, D. M. & W. H. BELDA. 1944. *Biol. Bull.* **87**: 138-144.
38. PACE, D. M. & T. E. KIMURA. 1946. *Proc. Soc. Exptl. Biol. Med.* **62**: 223-227.
39. PACE, D. M. & B. W. McCASHLAND. 1951. *Proc. Soc. Exptl. Biol. Med.* **76**: 165-168.
40. PACE, D. M. & B. L. FROST. 1952. *Biol. Bull.* **103**: 97-103.
41. REICH, K. 1955. *Physiol. Zool.* **28**: 145-151.
42. SUSCA, L. A. & C. G. WILBER. 1949. *Federation Proc.* **8**: 153.
43. COHN, B., R. CHAMBERS & P. REZNIKOFF. 1928. *J. Gen. Physiol.* **11**: 585-612.
44. HULPIEU, H. R. 1930. *J. Exptl. Zool.* **56**: 321-361.
45. PANTIN, C. F. A. 1930. *Proc. Roy. Soc. London.* **B105**: 538-554.
46. PANTIN, C. F. A. 1930. *Proc. Roy. Soc. London.* **B105**: 555-564.
47. SEAMAN, G. R. 1947. *Trans. Am. Microscop. Soc.* **66**: 212-218.
48. WILBER, C. G. & G. R. SEAMAN. 1946. *Anat. Record.* **96**: 500.
49. NARDONE, R. M., D. C. BRAUNGART, M. P. HURLEY & M. E. GILSON. 1956. *J. Protozool.* **3**: 119-121.
50. ORD, M. J. & J. F. DANIELLI. 1956. *Quart. J. Microscop. Sci.* **97**: 17-28.
51. ORD, M. J. 1956. *Quart. J. Microscop. Sci.* **97**: 39-45.
52. SCHAEFFER, A. A. 1917. *J. Exptl. Zool.* **22**: 53-86.
53. MARSHAK, A. 1944. *J. Gen. Physiol.* **28**: 95-102.
54. KASSEL, R. & M. J. KOPAC. 1953. *J. Exptl. Zool.* **124**: 279-301.
55. KASSEL, R. & M. J. KOPAC. 1954. *J. Exptl. Zool.* **126**: 497-509.
56. HOLTER, H. & J. M. MARSHALL, JR. 1954. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **29**: 7-26.
57. CHAPMAN-ANDRESEN, C. & H. HOLTER. 1955. *Exptl. Cell Research. Suppl.* **3**: 52-63.
58. ANDRESEN, N., C. CHAPMAN-ANDRESEN & H. HOLTER. 1952. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **28**: 189-220.
59. CHAPMAN-ANDRESEN, C. & R. V. ROBINSON. 1953. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **28**: 343-357.
60. ANDRESEN, N. 1954. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **29**(26): 435-555.
61. GOLDSTEIN, L. & W. PLAUT. 1955. *Proc. Natl. Acad. Sci.* **41**: 874-880.
62. RABINOVITCH, M. & W. PLAUT. 1956. *Exptl. Cell Research* **10**: 120-124.
63. MAZIA, D. & D. M. PRESCOTT. 1955. *Nature.* **175**: 300-301.
64. BRACHET, J. 1955. *Pubbl. staz. zool. Napoli.* **27**: 146-159.
65. BRACHET, J. 1955. *Nature.* **175**: 851-853.
66. BRACHET, J. 1956. *Exptl. Cell Research.* **10**: 255-256.
67. MARSLAND, D. A. & D. E. S. BROWN. 1942. *J. Cellular Comp. Physiol.* **20**: 295-305.
68. Ts'o, P. O. P., J. BONNER, L. EGGMAN & J. VINOGRAD. 1956. *J. Gen. Physiol.* **39**: 325-347.
69. Ts'o, P. O. P., L. EGGMAN & J. VINOGRAD. 1956. *J. Gen. Physiol.* **39**: 801-812.
70. WEBER, H. H. 1955. *The Harvey Lectures. 1953-1954.* : 37-56. Academic Press. New York, N. Y.
71. WEBER, H. H. 1955. *Symposia Soc. Exptl. Biol.* **9**: 271-281.
72. KRISZAT, G. 1949. *Arkiv Zool.* **1**: 81-86.
73. KRISZAT, G. 1954. *Arkiv Zool.* **6**: 195-201.
74. GOLDACRE, R. J. & I. J. LORCH. 1950. *Nature.* **166**: 497-500.
75. ZIMMERMAN, A. M., J. V. LANDAU & D. A. MARSLAND. 1958. *Exptl. Cell Research.* **15**: 484-495.

76. BRACHET, J. 1952. *Experientia*. **8**: 347-349.
77. MARSLAND, D. A. & D. E. S. BROWN. 1936. *J. Cellular Comp. Physiol.* **8**: 167-178.
78. BROWN, D. E. S. & D. A. MARSLAND. 1936. *J. Cellular Comp. Physiol.* **8**: 159-165.
79. LANDAU, J. V., A. M. ZIMMERMAN & D. A. MARSLAND. 1954. *J. Cellular Comp. Physiol.* **44**: 211-232.
80. LANDAU, J. V., D. A. MARSLAND & A. M. ZIMMERMAN. 1955. *J. Cellular Comp. Physiol.* **45**: 309-329.
81. KRISZAT, G. & J. RUNNSTROM. 1951. *Trans. N. Y. Acad. Sci.* **13**(5): 162-164.
82. LINDBERG, O. 1950. *Exptl. Cell Research*. **1**: 105-114.
83. RUNNSTROM, J. & G. KRISZAT. 1950. *Exptl. Cell Research*. **1**: 284-303.
84. LOOMIS, W. F. & F. LIPPMAN. 1948. *J. Biol. Chem.* **173**: 807-808.
85. SIMON, E. W. 1953. *Biol. Revs.* **28**: 453-479.
86. CLOWES, G. H. A. 1951. *Ann. N. Y. Acad. Sci.* **51**(8): 1409-1431.
87. ZIMMERMAN, A. M., J. V. LANDAU & D. A. MARSLAND. 1957. *J. Cellular Comp. Physiol.* **49**: 395-435.

Part IV. Nuclear-Cytoplasmic Relationships

NUCLEAR CONTROL OF CYTOPLASMIC ACTIVITIES

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The study of enucleated amoebae or anucleate fragments is somewhat frustrating to the investigator who attempts to understand the after-effects of the treatment. The visible activities of the anucleate cell and the changes in cytoplasmic constituents do not indicate the profound damage done by removal of the nucleus. A consensus of early publications is nicely summarized by Minchin: "Non-nucleated fragments may continue to live for a certain time; in the case of *Amoeba* such fragments may emit pseudopodia, the contractile vacuole continues to pulsate, and acts of ingestion and digestion that have begun may continue; but the power to initiate the capture and digestion of food ceases, consequently all growth is at an end, and sooner or later all non-nucleated bodies die off" (F. M. Summers).

On the whole, there is general agreement with the above statement, except perhaps with that portion dealing with food capture and digestion. I have observed paramecia, damaged by neutral red, captured and "digested" in food vacuoles of anucleate fragments. The "digestion" however, could have been in reality autolysis.

Factors considered to be of importance in the activities and longevity of the fragment are the size of the fragment (thus an enucleated amoeba might be expected to have the longest life span and the maximal degree of activity as compared to an anucleate half of an amoeba), and the area of the amoeba removed. If the fragment is from the posterior half ("old gel area") the locomotor activities are reduced as compared with a fragment obtained from the anterior or pseudopod-forming region.

There is great need for the development of a chemically defined medium for the axenic culture of the large free-living amoeba. At present metabolic studies of aging anucleate populations become involved in questions of techniques of culture. Current comparative studies between nucleate and anucleate populations tend to be of the short-range type: terminated within 24 hours, or imposed conditions of starvation (for example, several washes and then maintenance in sterile salt solutions). The latter approach adds the effects of starvation to the effects of anucleation. It is based on the premise that a starving nucleate fragment is an adequate control for the anucleate fragment, which is not necessarily the case, as shown in some of the short-range studies. However, at present there is no other alternative, unless the techniques for the induction of pinocytosis (as reported by Marshall and by Holter in this monograph) can provide an approach to the problem of obtaining a well-defined medium for amoebae.

Possibly the most comprehensive reports on the activities of enucleate amoebae and anucleate fragments are those of Clark, 1942 and 1943. In his analyses of the behavior of the anucleate, Clark emphasized the importance of the nu-

cleus in the maintenance of "viscosity." He described the increased movements of granules in the vicinity of an ingested rotifer within the enucleate amoeba and correctly interpreted this as a decrease in gel strength. Since this was within a short time after enucleation, Clark postulated an immediate dependence of the viscosity on the nucleus. The similarity of the corrugated surface of dividing amoebae to that of the newly enucleate organism was noted. Clark concluded that "The cause of ultimate death is due to a dedifferentiation of the ultrastructures normally present in the cytoplasm and maintained there by nuclear action" (1942). The results obtained in these studies fully support his observations on gelational changes. Clark's conclusion will be discussed later in this report.

The procedure employed for separating the amoebae into halves first involves the preparation of a suitable cutting tool. A tip of a glass rod about 5 mm. in diameter and about 8 inches in length is pulled so that a sharply tapered projection is obtained. The projection is needlelike and may be about 10 micra at its tip to about 1 mm. at the junction with the rod. The needlelike portion is trimmed down to about 3 cm. and then carefully brought horizontally over a microburner made from a hypodermic needle and allowed to heat slowly about 1 mm. from the tip. By careful control of the flame and the distance of the needle to the flame, an exact right angle bend can be produced, by gravity, at the point where the heat is applied. The bent tip, approximately 1 mm. in length, is the area used in the transection.

The amoebae to be used are placed, after several washes in sterile Brandwein solution, in a deep cell depression slide. The depression is completely filled with Brandwein solution. In this laboratory all of the manipulations are made by using a wide field dissecting scope and magnifications up to $\times 112.5$. After placing the amoebae in the depression slide, the microscope lamp is turned off to accelerate attachment and elongation. This takes from 15 to 30 min., depending upon the condition of the organisms.

The separation of the fragments is performed in the deep cell depression slide. The bent portion of the needle is pressed perpendicularly to the long axis of the amoeba. If the bend is at right angles, the pressure will separate the form into relatively equal halves. Selection can be made of anterior or posterior regions or mixtures of these regions by pressing when the nucleus is located in the appropriate area. It is probably simpler to obtain anucleate fragments by immediate selection of the observed area lacking the nucleus than to utilize the poor-attachment and light-response method of separation. In the latter case, unattached whole amoebae or damaged nucleate fragments may be pipetted out with the anucleate fragments; several separations are necessary to ensure a uniform population. In addition, the population left in the depression slide must similarly be sorted as to their being nucleate fragments or whole uncut amoebae. Another detriment is that in some instances the most active anucleate fragments may be overlooked. Utilizing either approach, however, one can obtain about fifty fragments within one hour.

As Clark (1942) has reported previously, an immediate transient blebbing occurs only within the anucleate fragment and can be observed to take place during the process of separating the whole amoeba into fragments. When

pressure from the needle is applied to the organism, that portion having the nucleus will produce pseudopods, whereas the anucleate region will exhibit surface corrugations or blebbings. These criteria can be used to select anucleate fragments, where the nucleus may be obscured, with astonishingly accurate results. An explanation for this occurrence is conjectural. The resemblance to the telophase animal cell has been noted by Clark. The blebbing might indicate sudden changes from the gel to the sol state in the cortical region. The artificially induced "furling" would correspond to the cleavage of cells. The involvement of the nucleus in this process further emphasizes the critical role of the nucleus in gelational states of the cell. On the other hand, the pressure on the cytoplasm may trigger the response reflected as pseudopod formation in the nucleate fragment and abortive pseudopod formation or "blebbing" in the anucleate fragment.

The activity selected for investigation and for this report was that of amoeboid movement, as expressed by the gelational state of the anucleate fragment. The Marsland pressure apparatus provides a useful means for measuring gelational strength (Marsland, 1950). Using the criterion of smooth rounding of the amoebae, Landau, *et al.* determined critical temperature-pressure relationships for *Amoeba proteus*. Their reported pressure values of 4000 psi (lb./in.²) at 15° C.; 5000 psi at 20° C.; and 5500 psi at 25° C. on whole amoebae have been confirmed in the course of this study. The effects of hydrostatic pressure on anucleate fragments were studied with A. Zimmerman and D. Marsland (for methods and details see Hirshfield *et al.*, 1958a). The results of those studies are presented in TABLE 1 and are plotted in FIGURE 1. The data show that the gel strength of the nucleate halves is virtually the same as that of the whole amoebae. On the other hand, the anucleate fragments have

TABLE 1

Temperature (Centigrade)	Pressure lb./in. ²	Percentages of fully rounded specimens		
		Nucleate halves	Anucleate halves	Whole amoebae
15°	2000	2 (48)*	25 (98)	—
	2500	5 (76)	67 (83)	4 (80)
	3000	22 (102)	98 (50)	16 (200)
	4000	69 (90)	—	75 (224)
20°	2000	—	8 (51)	—
	3000	8 (63)	61 (106)	7 (98)
	4000	33 (96)	98 (62)	36 (213)
	5000	73 (78)	100 (40)	77 (221)
25°	3000	2.5 (80)	35 (156)	2 (50)
	4000	8 (64)	62 (93)	9 (100)
	5000	59 (30)	95 (100)	61 (218)
	5500	68 (90)	100 (40)	74 (68)
	6000	93 (70)	—	89 (230)

* Figures in parentheses indicate total numbers of specimens observed in each case. Fractional percentages omitted, that is, percentages, represent nearest whole numbers (Hirshfield *et al.*).

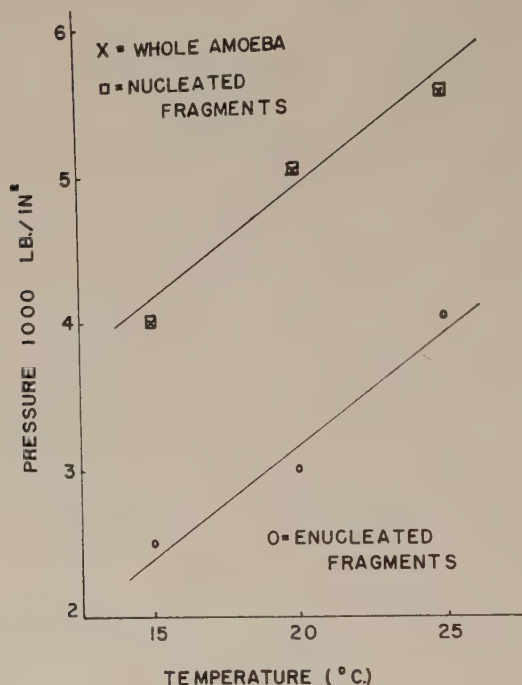


FIGURE 1. Comparison between nucleate and anucleate specimens of *A. proteus* in regard to the effect of temperature upon the minimum pressure required to produce total rounding up (solation).

considerably lower gel strength at the selected temperatures. The measurements can be made at about 2 hours after separation of the whole amoeba into the halves. The results confirm previous observations of the immediate change of lowered viscosity in anucleate fragments and enucleate amoebae. The anucleate fragments resume their characteristic shape and motility upon their return to atmospheric pressure. No visible effects of the temporary exposure to higher pressures was observed 24 hours after the treatment.

The response of the anucleate fragment to the temperatures selected is of interest. The slope of the graph in FIGURE 1 for the anucleates is the same as that for whole amoebae. The gel strength can be increased within biological limits by increments of heat (temperature). The results emphasize the importance of energy availability to the cytoplasm for gel maintenance. The anucleate gel strength at 25° C. equaled that of whole amoebae at 15° C.

The effects of selected temperatures on the survival of anucleate fragments were studied by Lynch, who found that the survival of the anucleates was higher at the lower temperatures (10° C.) than at the higher temperatures selected (20° C., 30° C.). Lynch's observations seem to be the reverse of those obtained with the effects of pressure. The two studies, however, are consistent with present knowledge. The anucleate fragments at 25° C. with the gel

externally stiffened were probably not receiving energy in a form utilizable for their metabolism. The acceleration of metabolic processes at this temperature imposed a more rapid exhaustion of available energy than at lower temperatures, and thus decreased length of survival. This interpretation is supported by the effects of ultraviolet radiation on anucleate fragments.

Mazia and Hirshfield (1951) showed that the survival of anucleate fragments could be reduced by exposure to 254 m μ ultraviolet (Sterilamp) radiation. The anucleate fragments were more affected by the exposure than were the nucleate fragments or whole amoebae. The observations of these investigators were confirmed by Skreb-Guilcher and Errera (1957). Further confirmation was made in this laboratory by D. Frisch, using monochromatic ultraviolet radiation. Frisch found the most effective wave length was 254 m μ . The survival of the anucleate fragments is thus intimately associated with nucleic acid metabolism.

The effects of monochromatic ultraviolet radiation on the gel strength of whole *A. proteus* were studied in this laboratory with A. Zimmerman and D. Marsland (1958b). Additional studies were done on anucleate fragments of *A. proteus* with J. Liberatore. Two hundred eighty m μ was found to be the most effective wave length in reducing gel strength (TABLE 2) in both the fragments and whole amoebae. The structural protein is thus intimately associated with gel maintenance.

Cytoplasmic dependence on the nucleus for phosphate metabolism was shown by Mazia and Hirshfield (1950) and, since then, this finding has been confirmed by other investigators, especially Brachet (1952). Brachet (1950) and Linet and Brachet (1951) showed a similar dependence of ribonucleic acid (RNA) upon the nucleus for *A. proteus* (see also Brachet in this monograph).

Survival of the anucleate fragments can thus be placed in the framework of nuclear control of nucleic acid metabolism and the motility of the anucleate fragment in the framework of nuclear control of cytoplasmic protein maintenance.

The available evidence leaves little doubt as to the primary role of the nucleus in the following cytoplasmic activities: phosphate turnover, protein gel-sol transformations, and RNA levels. There is similar evidence of a residual

TABLE 2
PRESSURE REQUIRED* TO INDUCE >50 PER CENT ROUNDING

Temperature centigrade	Whole amoebae and nucleate fragments			Anucleate fragments		
	Unirradiated	6000 ergs/sec./mm. ²		Unirradiated	4000 ergs/sec./mm. ²	
		265 m μ	280 m μ		265 m μ	280 m μ
	psi	psi	psi	psi	psi	psi
15°	4000	—	—	2500	—	—
20°	5000	5000	4000	3000	3000	2500
25°	5500	—	—	4000	—	—

* Lb./in.²

cytoplasmic capability of functioning, although at a reduced level, in the absence of the nucleus. In the case of phosphate, there was uptake, although greatly diminished, of P^{32} (Mazia and Hirshfield). As previously noted, the gel strength of the anucleate fragment was further reduced by exposure to 280 m μ ultraviolet radiation (Hirshfield and Liberatore). Plaut presents evidence elsewhere in this monograph that RNA can be synthesized by anucleate fragments. In the above activities the nucleus may function primarily as the maintainer of the functional equilibria, or in steady state control (Hirshfield *et al.*, 1958a).

The imposed strengthening of anucleate plasmagel by the addition of heat increments has been discussed. Preliminary studies on the gel-strength response of anucleate fragments to adenosine triphosphate (ATP) have been undertaken in this laboratory (Hirshfield and Liberatore). The concentration tested, 0.0005 M, has been found to strengthen gel in whole amoebae. (See Zimmerman). Our results, using anucleate amoebae fragments, while not conclusive, indicated a similar response of the anucleates, 40 per cent of the population rounding, as opposed to 55 per cent of the untreated controls, at 3000 psi and 20° C. There is thus an indication that the proteins in an area deprived of a nucleus still retain, at least for a short duration, means of utilizing an adequate energy source. This is substantiated by a variety of observations on the relative lack of effect on cytoplasmic enzymes by the removal of the nucleus (Holter and Kopac, Brachet, and others).

The investigations of Brachet and his co-workers (1955) have indicated that ATP and ATPase levels in anucleate fragments remain substantially the same as in nucleate fragments. One possible explanation of the results obtained in this laboratory for the ATP effect of strengthening the plasmagel is the availability of the ATP to the proteins involved. In this respect, it is inviting to postulate a nuclear-dependent, cytoplasmic structure based on recent electron microscopic studies of amoebae. Cell fine studies show the presence of what might be interpreted as channels between the nuclear membrane and the cytoplasm (Pappas). Should these channels and the nuclear membrane pores (Pappas) be in fact functional, the cell would possess a means of rapid transport between the interior of the nucleus and the periphery of the cytoplasm. The removal of the nucleus by damage, enucleation, or transection would irreversibly destroy the integrity of the system. This concept is by no means new as a means of explaining the effects of nuclear removal. Clark in 1942 stated essentially the same thing (see above). Further electron-microscopic studies of enucleate whole amoebae and anucleate fragments might be conclusive tests of the hypothesis.

The possibility that the nucleus might act as a means of supplying utilizable energy where needed by the cytoplasm is weakened by the studies of Brachet (1955), which show the presence of ATP in equivalent amounts in nucleate and anucleate fragments. The studies of temperature and ATP in this laboratory on anucleate fragments do not support the concept that the immediate effects of enucleation are due to the inability of the cytoplasmic proteins to utilize energy sources. This finding is also supported by observations of the synthesis of the nucleic acids in anucleates (Plaut). The gradual dwindling

of cytoplasmic RNA, while nuclear-dependent, cannot produce the immediate rapid change in gel strength. The RNA diminution, instead, might also be due to the destruction of the normal transport mechanism of the cell.

The data obtained in this laboratory indicate that the cytoplasmic protein component involved in motility (gel-sol transformations) can continue to function—poorly at best—in the absence of the nucleus.

Among the most significant questions still unanswered are those regarding the nature of the nuclear factors involved and their degree of dependence on cytoplasmic function. The relationship between nuclear and cytoplasmic nucleic acids is still largely conjectural, while little is known of the relationship between nuclear and cytoplasmic proteins. A possible relationship between one class of nuclear components and another should not be overlooked: for example, nuclear nucleic acid and cytoplasmic protein. Furthermore, these are but two of the many components of the cell.

Nuclear transplant studies pioneered by J. F. Danielli offer an exciting means of evaluating the relative importance of the nuclear role in cellular organization (structure maintenance) when used in conjunction with the electron microscope. Nuclear transplants, in conjunction with isotopic tracers, as reported by Plaut, may provide an equally interesting approach to an understanding of the nature of the relationship between the nucleus and the cytoplasm.

In summary: at least two distinctive kinds of effects of nuclear absence in amoebae can be distinguished at present: (1) a long range disturbance of nucleic acid metabolism leading gradually but irreversibly to the death of the cell and (2) an immediate and profound disturbance in the structural state of the cytoplasmic proteins associated with the motor activities of the cell.

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References

- BRACHET, J. 1950. Une étude cytochimique des fragments nucléés et enucléés d'amibes. *Experientia*. **6**: 294.
- BRACHET, J. 1952. Le rôle du noyau cellulaire dans les oxydations et les phosphorylations. *Biochim. Biophys. Acta*. **9**: 221-222.
- BRACHET, J. 1955. Recherches sur les interactions biochimiques entre le noyau et le cytoplasme chez les organismes unicellulaires. I. *Amoeba proteus*. *Biochim. Biophys. Acta*. **18**: 247-268.
- CLARK, A. M. 1942. Some effects of removing the nucleus from amoeba. *Australian J. Exptl. Biol. Med. Sci.* **20**: 241-249.
- CLARK, A. M. 1943. Some physiological functions of the nucleus in amoeba investigated by micrurgical methods. *Australian J. Exptl. Biol. Med. Sci.* **21**: 215-221.
- FRISCH, D. Effects of monochromatic ultraviolet irradiation on anucleate fragments of *Amoeba proteus*. Unpublished.
- HIRSHFIELD, H. & J. LIBERATORE. Unpublished.
- HIRSHFIELD, H., A. ZIMMERMAN & D. MARSLAND. 1958a. The nucleus in relation to plasmagel structure in *Amoeba proteus*; a pressure-temperature analysis. *J. Cellular Comp. Physiol.* In press.
- HIRSHFIELD, H., A. ZIMMERMAN & D. MARSLAND. 1958b. Effects of UV-irradiation on form and sensitivity to pressure-solation in *Amoeba proteus*. *Abstr. Anat. Record*.

- HOLTER, H. & M. J. KOPAC. 1937. Studies on enzymatic histochemistry. XXIV. Localization of peptidase in the amoeba. *J. Cellular Comp. Physiol.* **10**: 423-437.
- LANDAU, J. B., A. ZIMMERMAN & D. MARSLAND. 1954. Temperature-pressure experiments on *Amoeba proteus*; plasmagel structure in relation to form and movement. *J. Cellular Comp. Physiol.* **44**: 211-232.
- LINET, N. & J. BRACHET. 1951. L'évolution de l'acide ribonucléique et du glycogène dans des fragments nucléés et enucléés d'amibes. *Biochim. Biophys. Acta.* **7**: 607-608.
- LYNCH, V. 1919. The function of the nucleus of the living cell. *Am. J. Physiol.* **2**: 258-283.
- MARSLAND, D. 1950. The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cellular Comp. Physiol.* **36**: 205-227.
- MAZIA, D. & H. HIRSHFIELD. 1950. The nucleus-dependence of P^{32} uptake by the cell. *Science.* **112**: 297-299.
- MAZIA, D. & H. HIRSHFIELD. 1951. Nucleus-cytoplasm relationships in the action of ultraviolet radiation on *Amoeba proteus*. *Exptl. Cell Research.* **2**: 58-72.
- SKREB-GUILCHER, Y. & M. ERRERA. 1957. Action des rayons U.V. sur des fragments nucléés et anucléés d'amibes. *Exptl. Cell Research.* **12**: 649-656.
- SUMMERS, F. M. 1941. The protozoa in connection with morphogenetic problems. Chap. XVI. *In* Protozoa in Biological Research. : 787. G. N. Calkins and F. M. Summers, Eds. Columbia Univ. Press. New York, N. Y.

MICROTECHNIQUES IN AMOEBAE STUDIES

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From the viewpoint of the cell physiologist the large, free-living amoebae present many of the favorable characteristics of the so-called "ideal experimental cell." The two principal advantages of the amoeba, especially appreciated by the experimenter, are large size and exceptional ruggedness. The amoeba is readily handled, micromanipulated, and subjected to micrurgy with surprisingly minor traumatic effects. The single, serious experimental disadvantage of this cell type lies in the lack of understanding concerning its nutrient requirements. These are apparently complex, and culture methods are necessarily still quite crude. The possibilities for precisely controlled metabolic experiments are very limited.

The inadequacies of culture methods limit only certain types of experiments, and amoebae are extremely useful for the investigation of many problems in cell physiology. More is probably known about the physiology and growth of *Amoeba proteus*, for example, than any other particular cell type. Much of my own interest in this amoeba has centered around problems of cell growth and its relation to cell division, and I propose to discuss some of this work. Moreover, many people have attempted to grow the larger amoebae under axenic conditions. There are no reports of success, and a brief description of our own efforts in this direction will be given, even though the results are for the most part discouragingly negative.

Growth of Individual Amoebae

A basic requisite in the study of individual cell growth from division to division is adequate control over the cell's life cycle. Under a culture system for individual cells devised by James,¹ *A. proteus* consistently shows a generation time of 24 hours at 23° C. With this system, measurements on single cells in isolated culture can always be related to both the preceding and forthcoming cell division.

With the Cartesian diver balance,² the weights of living tissues and cells can be determined under physiological conditions that avoid injury to the living material. The weighing of the large, multinucleated *Pelomyxa*,³ for example, is a relatively simple task. By reducing the scale of the diver balance by approximately another one hundredfold, we have an instrument that permits an accurate weight determination of a single living *A. proteus*.⁴ The sensitivity of such a diver balance is about 10^{-10} gm. Such very small diver balances are difficult to calibrate, and the absolute weight of an amoeba can be determined only with an accuracy of about ± 10 per cent, but the relative error in comparing one weighing with another is only about ± 1 per cent.

FIGURE 1 shows a single *A. proteus* in a diver balance just before division, during division, and shortly after cytoplasmic fission has been completed. The difficulties in working with the diver balance at this scale can perhaps be ap-



FIGURE 1. Photomicrographs of an *A. proteus* diver balance: (a) during the predivision period; (b) during division; and (c) at the completion of cytoplasmic fission.

preciated from the physical dimensions. The balance itself is about $400\ \mu$ across the cup and has a dry weight of only $2\ \mu\text{g}$. A series of special techniques was necessary to make the balances, and the construction of the first instrument required a full 2 months' work.

To obtain growth curves for a single amoeba, a new daughter cell was weighed, removed from the balance, and cultured in a separate Petri dish. The amoeba was reweighed periodically through the interphase, and the last weighing was made on the dividing cell. Of the 34 experiments started, only 10 were carried to completion. In the 24 unsuccessful experiments the amoebae were either broken during pipetting or lost in the flotation chamber for the diver balance. In 2 instances, the diver balance was broken.

Successful growth curves for 6 individual amoebae are shown in FIGURE 2. In these cases the amoebae divided at the end of the predicted 24-hour period.

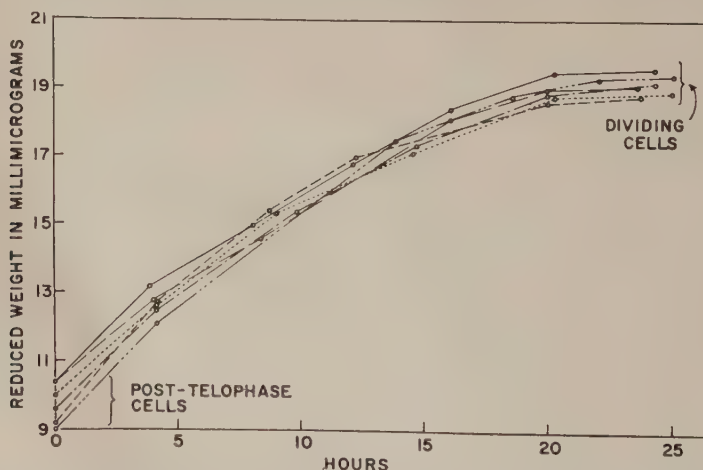


FIGURE 2. Growth curves in weight for 6 individual *A. proteus* over the cell division cycle at 23°C . Each curve begins with the weight of a daughter amoeba and ends with the weight of the same amoeba as it enters division.

In some of the completed but unsuccessful experiments the divisions were delayed far beyond the 24-hour period, and these data, although of some interest, could not be accepted as representing the normal growth picture. Each curve in FIGURE 2 begins with the weight of a newly divided amoeba and ends with the division of it. The 2 most notable characteristics of these curves are: (1) the almost linear pattern of growth denoting a constant, rather than an exponentially increasing, growth rate; and (2) the failure of the amoeba to increase in weight during the last few hours of the interphase. This phenomenon is consistent, and in no case has an amoeba been observed to continue its growth during the last few hours preceding prophase. Amoeba volume and protein also increase according to the pattern of weight increase.⁴

This predivision period of no growth reflects, of course, a period of very little or no food capture. In the case of amino acids dissolved in the medium, uptake appears to be governed by the incorporation rate of the amino acids into cellular proteins.⁵ It is also very likely that the rate of particulate food uptake (which is, after all, a form of active transport across the cell membrane) depends ultimately upon incorporation requirements within the cell. Therefore, the rate of food capture is simply a measure of the particular physiological conditions existing within the amoeba. The failure of food uptake during predivision cannot be due to an impairment of feeding mechanisms imposed by physical size; the large, binucleated cells, for example, are active feeders.

Many of the older theories on cell division have attempted to relate cell growth or size to the initiation of cell division. More specifically, these theories have considered as critical some factor such as the cytoplasmic-nuclear volume ratio, cell surface-cell volume ratio, or cell surface-nuclear volume ratio. None of these "dimension-ratio" schemes has been generally satisfactory, and recent experiments, particularly with protozoa, strongly tend to emphasize the lack of any direct dependence between division and size. Genetically identical cells differ markedly in size under different culture conditions without directly influencing cell division.

In the individual amoeba growth curves, a 4-hour period separates the end of growth from the onset of cell division. Certainly in this case there can be no direct connection between completion of growth (or attainment of some particular cell size) and the initiation of the division mechanisms. It is a fact, however, that nuclear volume increases most rapidly in amoebae during the predivision period of no growth.⁴ This information suggests that the nucleus may carry the prime control for setting up a cell division. In any case, the events that immediately control cell division are not reflected directly in the over-all growth pattern. This information would suggest that the cell cycle is composed of two concurrently advancing processes: (1) growth, which guarantees a doubling in cell size over the cycle; and (2) a series of synthetic steps, the completion of which brings the cell to the division state and is, thus, responsible for a doubling in cell number. A similar separation of growth and division is quite pronounced in the experiments of Scherbaum and Zeuthen⁶ on division synchronization of *Tetrahymena*. In this instance, with the use of heat shocks, the separation is so complete that growth continues with no intervening cell divisions, and giant cells are produced that divide synchronously

when the treatments are stopped. Admittedly, the *Tetrahymena* are in an abnormal state, but the experiments still point up the flexibility of the cell growth-division interdependence.

Although size is not immediately related to division in the amoeba, the length of the interphase period has a dependence upon cell size. If the weights of newly divided cells are compared to the generation times, a definite relationship is apparent. Smaller cells require more time to reach division and vice versa. The variation in the weights of newly divided amoebae is normally not very great. Dividing amoebae subjected to strong light, however, divide unequally, and the variation in cell sizes is greatly increased. The relationship between daughter cell weight and subsequent generation time is illustrated by the data in FIGURE 3. There are actually 2 different relationships, since a particular weight change in one direction has a different effect from that of the same change in the opposite direction. For example, a cell 40 per cent smaller than normal size has a generation time increased by 35 per cent, while a cell 40 per cent larger than normal has a generation time shortened by only about 17 per cent. In other words, a given size reduction is twice as effective in retarding the progress of a cell toward division as an equivalent size increase is in accelerating the process. The reasons for the relationships are not known, but at least the data demonstrate that the rate at which an amoeba approaches division is in some way dependent upon cell size.

The extreme case of size shift can be accomplished by inhibiting completely cytoplasmic fission and micrurgically removing one of the two newly reconstituted nuclei. Such a cell would be composed of a cytoplasmic mass equivalent to that of a cell about to divide, but containing a nucleus that has just completed mitosis. The production of binucleates can be accomplished by subjecting dividing amoebae to various treatments such as simple mechanical shaking, exposure to strong light, or transfer to distilled water.⁷ Binucleates can be produced most consistently, however, by exposing dividing amoebae to a 1 per cent solution of egg albumin for about 1 hour. The relatively long exposure is necessary because treated cells remain capable of division for about 20 min. after untreated controls have completed division. A protein solution induces pinocytosis in amoebae, but dividing amoebae do not show this reaction. Removal of one of the nuclei is accomplished easily with a glass loop just large enough to surround a nucleus; this loop is controlled by a micro-manipulator. A few of these division-size cells, each with a single postdivision nucleus, have been studied. For a short time following the removal of one of the nuclei, amoeboid movement is somewhat impaired. Pseudopod formation is reminiscent of an enucleated amoeba. Feeding is very sluggish, and growth is slight at most. Whether the amoebae are permitted to feed, however, does not influence the length of the succeeding interphase, and these cells divide at the end of 14 and 18 hours. These data are represented in FIGURE 3 by the daughter cells with weights of approximately 20 μg . The most notable thing about these cells is the fact that, although they are of division size, they require 60 to 75 per cent of a normal interphase to reach division. This length of interphase is perhaps the time required by a fully grown cell to complete the nongrowth series of synthetic steps that lead to cell division.

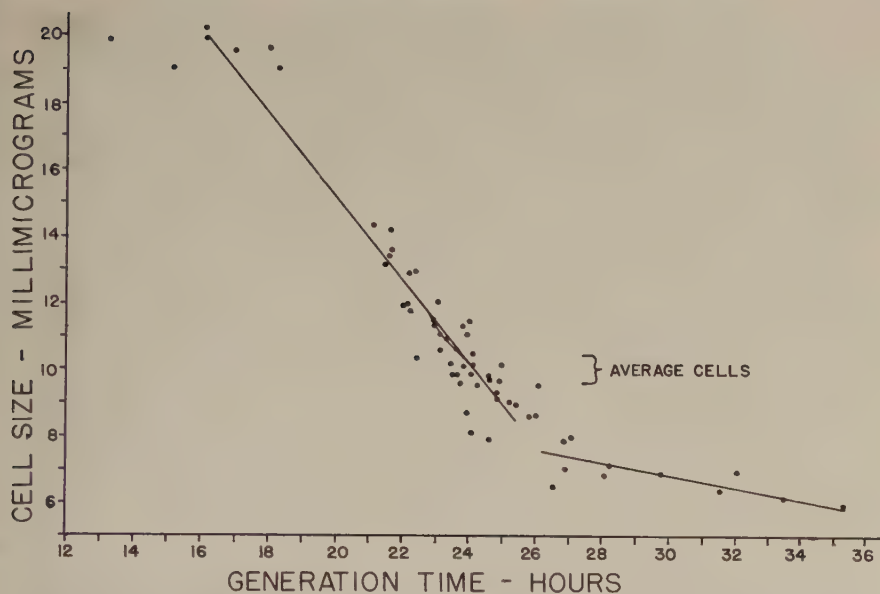


FIGURE 3. The relation between daughter cell size and generation time. Each point in the graph represents the weight of a single daughter amoeba plotted against its subsequent generation time.

These experiments raise a number of obvious biochemical questions concerning the time relations in the cell cycle of desoxyribonucleic acid synthesis, ribonucleic acid synthesis, and protein accumulation within the nucleus. In amoebae, unfortunately, experiments of this type will be very difficult, if not impossible, until a completely defined culture medium is devised.

Culturing of Amoebae

The simplest and most efficient culture system for *A. proteus* and *Pelomyxa* contains *Tetrahymena* and small numbers of bacteria and yeasts.⁸ The *Tetrahymena* serve as the main source of nutrients for the amoebae, while the bacteria (and perhaps yeasts) supply essential growth factors not present in the *Tetrahymena*. These cultures succeeded so well that, initially, it was considered that *Tetrahymena* might provide all the amoeba's nutrient requirements. A 2-membered culture containing living *Tetrahymena* and amoebae, but absolutely no bacteria or yeasts, does not result in any proliferation of amoebae. The mixed culture system, however, provides large numbers of amoebae for experimentation that can be easily washed free of contaminating organisms. A Petri dish 9 centimeters in diameter will produce about 1 gm. of amoebae (about 1,000,000 *A. proteus*). The cultures can be grown on either glass or nonnutrient agar. FIGURE 4 is a photograph of a young amoeba culture.

In attempting to devise a complete culture medium for *A. proteus*, it is essential to have a ready supply of amoebae completely free of all other living organisms. The *Tetrahymena* and yeasts can be removed by the simple proc-

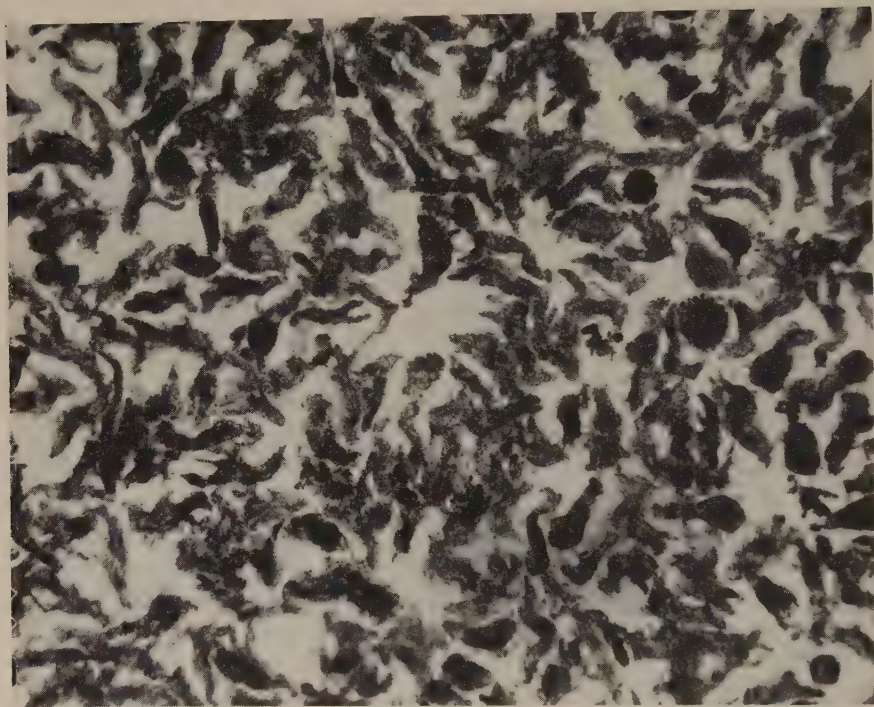


FIGURE 4. A photomicrograph of a section of an *A. proteus* culture in which *Tetrahymena* serve as the principal food source. Twenty-four hours have elapsed since the last addition of food organisms.

ess of washing. The bacteria present a more difficult problem, but they can be eliminated by 3 or 4 transfers of the amoebae over a 2- or 3-day period through mixed solutions of penicillin and Aureomycin (both at a concentration of 1 mg./5cc. of sterile inorganic medium). Streptomycin is toxic to amoebae even at very low concentration. We have tested a number of media with negative results. Proteose peptone, which supports so well the growth of other protozoa (including *Tetrahymena* and *Acanthamoeba*), is very toxic to *A. proteus* and *Pelomyxa*. Equally toxic are bacto-peptone, yeast extract, yeast autolysate, and bactosoytone. A 0.3 per cent solution (w/v) of liver extract,* is the only medium that has given any encouragement; although it does not support growth, it nevertheless increases the longevity of amoebae over starving controls. Extracts of various plant materials such as oatmeal, barley, rice, and lettuce do not promote growth. Even homogenates of amoebae themselves do not support growth and, at higher concentrations, they are very toxic. The initial problem has been to find a medium which, although it may not support growth, is at least nontoxic.

* Nutritional Biochemical Corp., Cleveland, Ohio.

The resistance of *A. proteus* to our attempts to obtain axenic cultures is certainly discouraging. The extreme experimental usefulness this cell would have if grown axenically is sufficient incentive for a continued search. Currently, we are isolating strains of bacteria from rapidly growing amoeba cultures in the hope of finding a single bacterial type that will supplement *Tetrahymena* in providing all of the required growth factors. From such a system, graduation to *Tetrahymena*-bacteria homogenates and axenic cultures might be possible.

References

1. JAMES, T. W. 1953. Quantitative studies on the ribonucleic acid content in *Amoeba proteus* in relation to the nucleus and the division cycle. Ph.D. Thesis. Univ. Calif. Berkeley, Calif.
2. ZEUTHEN, E. 1948. A Cartesian diver balance weighing reduced weights (R.W.) with an accuracy of ± 0.01 micrograms. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **26**: 243-266.
3. HOLTER, H. & E. ZEUTHEN. 1948. Metabolism and reduced weight in starving *Chaos chaos*. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **26**: 277-296.
4. PRESCOTT, D. M. 1955. Relations between cell growth and cell division. I. Reduced weight, cell volume, protein content, and nuclear volume of *Amoeba proteus* from division to division. Exptl. Cell Research. **9**: 328-337.
5. MAZIA, D. & D. M. PRESCOTT. 1955. The role of the nucleus in protein synthesis in *Amoeba*. Biochim. Biophys. Acta. **17**: 23-34.
6. SCHERBAUM, O. & E. ZEUTHEN. 1954. Induction of synchronous division in mass cultures of *Tetrahymena piriformis*. Exptl. Cell Research. **6**: 221-227.
7. CHALKLEY, H. W. 1951. Control of fission in *Amoeba proteus* as related to the mechanism of cell division. Ann. N. Y. Acad. Sci. **51**(8): 1303-1310.
8. PRESCOTT, D. M. 1956. Mass and clone culturing of *Amoeba proteus* and *Chaos chaos*. Compt. rend. trav. Lab. Carlsberg, Sér. chim. **30**(1): 1-12.

MICRURGICAL STUDIES ON IRRADIATED *PELOMYXA**

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Micrurgical studies of individual cells and portions of them have been made on the giant amoeba, *Pelomyxa illinoisensis*, to gain more information about what intracellular structures and functions are critically damaged by radiation. At the same time, an effort has been made to find specific intracellular components that will prevent death after lethal radiation exposure. In earlier studies it was demonstrated that nonirradiated protoplasm prevents death after its microfusion into lethally X-irradiated amoebae.¹ Ord and Danielli² have shown in studies with *Amoeba proteus* that a nonirradiated nucleus can prevent death if put into a lethally irradiated cell. Subsequently, efforts in this laboratory have been made: (1) to find out whether or not nonirradiated protoplasm can also prevent death in cells lethally exposed to other types of radiation; and (2) to test the therapeutic capacity of different centrifuged strata taken from living pelomyxae and fused into supralethally X-irradiated cells³ and supralethally neutron-irradiated cells.⁴

Studies were also made to determine whether one type of radiation incapacitates the same component(s) as another type of radiation. To do this, lethally X-irradiated amoebae were combined with lethally neutron-irradiated cells on one hand, and with lethally ultraviolet-irradiated cells on the other. These results are presented in this paper and some theoretical considerations are put forth in the discussion.

MATERIALS AND METHODS

The organisms and culture methods have been described.^{5, 6} The present work was done on single cell isolates at room temperature ($24 \pm 1^\circ \text{C.}$). Some details of X-ray exposure^{1, 11} and of gamma-ray and fission-neutron exposure have been described.⁴ A Co^{60} source was used for the γ -irradiations. The exposures to neutrons were made at the Argonne CP-5 research reactor where the γ -ray component of the neutron beam was 15 to 20 per cent. The dose rates and some of the total dose values are shown in TABLE 1.

The methods used for centrifugation have been described previously.^{7, 11} The time of each centrifugation run was 10 min., exclusive of acceleration and deceleration, while the gravitational forces used in different runs ranged from 700 to 30,000 g. Stratified cells were put immediately into ice cold water after removal from the cold centrifuge tubes and then, within 20 min. or less, cut into halves or thirds. These portions were then injected into irradiated cells by fusion⁸ and the fused protoplast was cultured until it either died or produced a mass culture of about 20 amoebae.

The source of ultraviolet radiation was a General Electric 15-watt germicidal mercury vapor lamp with a peak emission at 2537 Å. A Latarjet dosimeter

* The work described in this paper was performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

TABLE 1
SENSITIVITY OF *PELOMYXA ILLINOISENSIS* TO DIFFERENT RADIATIONS

Dose rate	X rays	γ rays	Fission neutrons	Ultraviolet (2537Å)
	4 kr/min.	25r/min.	31 rep/min.	14.3 ergs sec. ⁻¹ mm. ⁻²
Approximate maximum dose producing no mortality	7 kr	9 kr	8 krep	<500 ergs mm. ⁻²
Approximate dose producing 50% mortality*	11 kr	17 kr	11 krep	2,150 ergs mm. ⁻²
Minimum dose which killed all cells* (LD ₁₀₀)	14 kr	25 kr	15 krep	5,000 ergs mm. ⁻²
Mean time of death in days after above dose (LD ₁₀₀)	4.9 \pm 0.2† (63)§	4.2 \pm 0.4 (25)	4.4 \pm 0.1 (25)	14.7 \pm 1.1 (50)
Supralethal dose used*	24 kr	40 kr	30 krep	13,000 ergs mm. ⁻²
Mean time of death in days after supralethal dose	4.6 \pm 0.1 (164)	4.1 \pm 0.2 (25)	4.2 \pm 0.1 (158)	5.0 \pm 0.2 (20)

* Period of observation was 10 days after ionizing radiation exposures and 45 days after ultraviolet irradiation.

† Standard error.

§ Numbers in parentheses represent numbers of cells observed.

was used to determine the output before each exposure. This dosimeter is made up of a selenium block layer photocell covered with a fluorescent layer with proper filters. The calibration of this device had a 5-per cent error. At a target distance of 53 cm., 50 organisms at a time were exposed in about 1 ml. of phosphate buffer.⁹ The organisms were spread out on the bottom of glass vials 6 mm. high that were open at the top to permit free transmission of the ultraviolet radiation. The pH of the medium was 6.9 to 7.0 and the temperature was kept between 22.5 and 23.5° C. during and after exposure. The cells were removed and isolated in fresh buffer medium about 15 min. after irradiation. All cells except those in a photoreactivation study were kept either in the dark or in red light. Although it was necessary to keep the experimental cells for only 10 to 15 days after exposure to ionizing radiation, those exposed to ultraviolet radiation were studied for 45 days, since some of the cells lived about this long before either mass culture formation or death.

RESULTS

Effect of Various Ionizing Radiations

The sensitivity of *P. illinoisensis* to different ionizing radiations (X rays, γ rays, fission neutrons) is shown in TABLE 1. The approximate doses producing 0, 50, and 100 per cent mortality, as well as doses that were considerably above those required to kill all cells, are shown for each type of radiation. The length of life of lethally irradiated amoebae was about the same whether a single lethal dose or a supralethal dose was given. Similarly, the amoebae that died after being exposed to an approximate LD₅₀ dose died after about the same lapse of time. Most of the amoebae that received a lethal dose during exposure to 16.5, 18, and 21 kr. of γ rays died between the fourth and fifth

postirradiation days. Similarly, of pelomyxæ receiving an LD_{50} dose of neutrons, the nonsurvivors showed survival times that were either within a 4- to 5-day postradiation interval or close to it. However, these moribund amoebæ did not show evidence of radiation damage during the first day after exposure to any of these ionizing radiations, except for the first few hours after X-irradiation, when they became rounded due to the retraction of pseudopodia.

Cell division rarely occurred in amoebæ that received either a single lethal dose or a supralethal dose of any of the ionizing radiations used. On the other hand, the nonsurviving cells among those given an LD_{50} dose divided once with higher probability (<40 per cent), but daughter cells of these amoebæ died without undergoing a second division. Most of the amoebæ that underwent two successive divisions after exposure to ionizing radiations gave rise to mass cultures. Thus, a single cell division does not determine whether or not the cell or its daughters will survive. The probability of survival after an LD_{70} X-ray dose among animals that had divided once was 52 per cent.¹⁰ Cell division in a lethally X-irradiated amoeba of this type has no influence on the death time of the clone. The 2 functions, however complex, appear to be independent.

Effect of Ultraviolet Radiation

The sensitivity of this amoeba to ultraviolet radiation is shown in TABLE 1 and FIGURE 1. The lethal dose was obtained after 5.5 to 6 min. exposure or about 5000 ergs $mm.^{-2}$. Although sufficient data are not available to differentiate between a linear and a sigmoid curve, the linear type curve is more closely approximated after ultraviolet radiation than after any of the ionizing radiations that have been used.

The mean survival time of amoebæ exposed to supralethal doses of ultraviolet radiation is 5 days or about the same as that after a killing dose of ionizing radiation (TABLE 1). These data are supported by studies of 200 amoebæ kept in mass cultures until death following a supralethal ultraviolet dose of 13,000 ergs $mm.^{-2}$, and also on death time measurements of 50 isolated amoebæ following a dose of 8600 ergs $mm.^{-2}$ at the rate shown in TABLE 1. On the other hand, after a dose of ultraviolet radiation just sufficient to kill all cells, the amoebæ survive about 15 days, or about 3 times longer than those given supralethal doses of ultraviolet radiation or any of the doses of ionizing radiations sufficient to cause some cells to die (TABLE 1). Amoebæ that die following ultraviolet doses near the LD_{50} also live about 15 days, or about 3 times as long as those given supralethal doses of the same radiation. On the other hand, the cells that die after exposure to doses of ionizing radiation near the LD_{50} die in about 5 days.

Cells lethally irradiated by UV frequently became gradually smaller in size until, at the time of death several days after irradiation, they were greatly reduced in size. These changes were not seen in the pelomyxæ dying after exposure to lethal doses of ionizing radiation. The lethally ultraviolet-irradiated amoebæ fed poorly if at all on other protozoa, in contrast to amoebæ exposed to lethal doses of ionizing radiations.

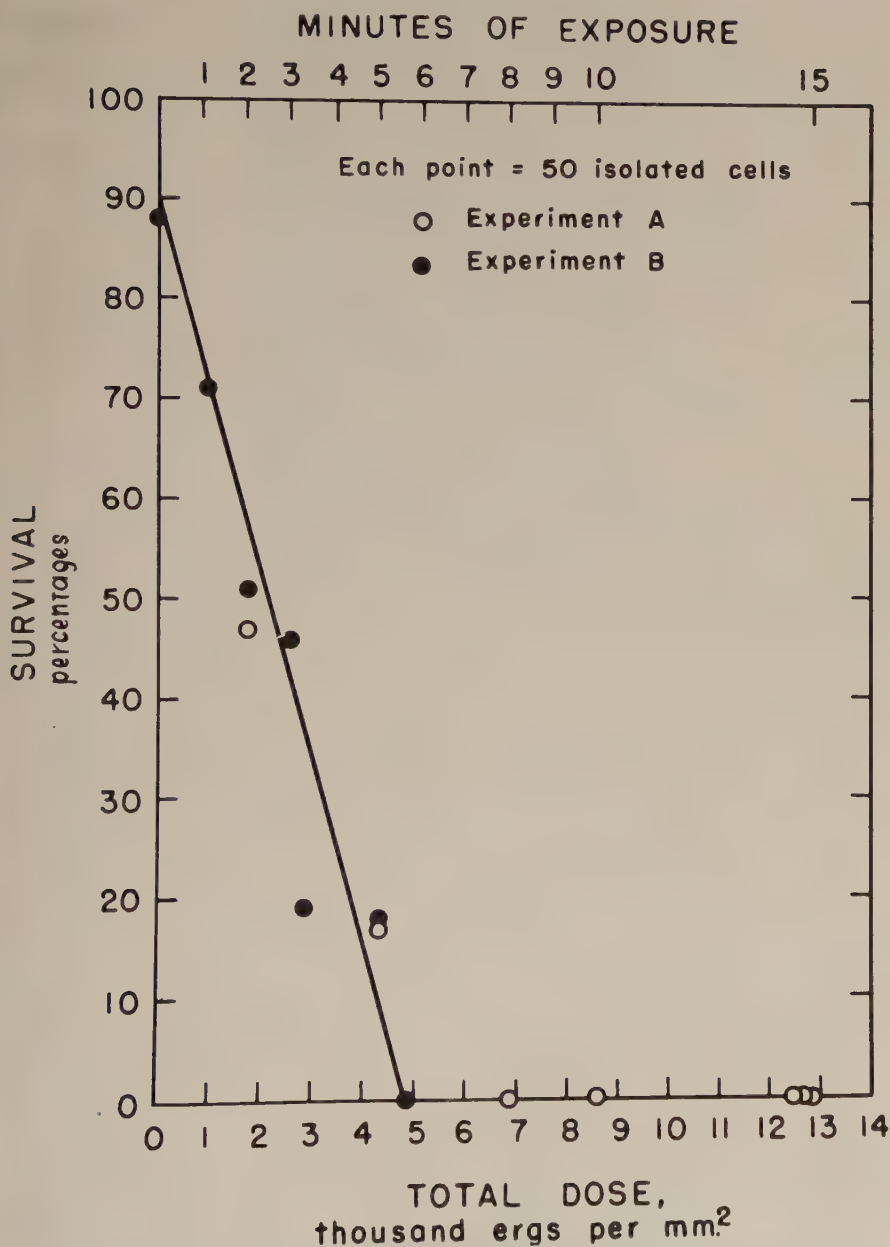


FIGURE 1. Survival curve of *Pelomyxa illinoensis* after exposure to ultraviolet radiation (2537 Å). Exposure rate = $14.3 \text{ ergs sec.}^{-1} \text{ mm.}^{-2}$

Amoebae exposed to a lethal or supralethal dose of ultraviolet radiation seldom underwent a cell division. If exposed to lower doses near the LD_{50} , 11 per cent of the single cell isolates that eventually died divided at least once and, among those that divided, 33 per cent had daughter cells that underwent one or more cell divisions. As previously mentioned, amoebae that underwent a second cell division (division of the daughter cell) after exposure to ionizing radiation almost always produced mass cultures. Among the survivors the mean time lapse between ultraviolet exposure and the first cell division was as long as or longer, but in no case shorter, than the mean time lapse between the first and second cell divisions. Four dose levels (955, 1715, 2568, and 4290 ergs $mm.^{-2}$) were studied (FIGURE 1). The actual time required for the first and second divisions was about twice that of the respective nonirradiated controls. The mean time between the second and third divisions in the irradiated amoebae was only slightly delayed.

Photoreactivation

In a study of photorecovery in *P. illinoisensis*, an LD_{50} dose (1715 ergs $mm.^{-2}$) of far ultraviolet was used to irradiate 2 groups of amoebae at the same time under similar conditions. One group (53 cells) was put in the dark after exposure and examined under red light thereafter to minimize photoreactivation. The other group (51 cells) was immediately exposed to white light continuously for 6 hours while being kept at constant room temperature. This light, emitted by a 100 w., 120 v., 60-cycle bulb in a microscope lamp (open diaphragm), was reflected by the ground-glass mirror of the microscope into the amoebae that were on the microscope stage. The distance of the light path was 12 cm. Both groups of amoebae were transferred to fresh media and isolated at approximately the same time. They were then subcultured daily until death or formation of mass cultures, which required 45 days in a few cases. Survival in the group that was not exposed to light was 51 per cent; that in the group exposed to white light was 65 per cent. According to Chi-square analysis of these data, this difference is not significant.

Therapeutic Effect of Nonirradiated Protoplasm

X-irradiated amoebae. Nonirradiated protoplasm prevents death when it is put into supralethally irradiated *P. illinoisensis* amoebae.^{1, 11} These data show that 92 per cent of 58 exposed cells survived after receiving nonirradiated protoplasm by fusion. Furthermore, cell division occurred in 95 per cent of the fused cells that survived 10 days, and test clones kept for longer periods produced mass cultures. This is similar to the occurrence of cell division in nonirradiated control cells.^{1, 4} Both irradiated and nonirradiated nuclei in a fused cell undergo the first mitosis after fusion simultaneously,¹¹ a phenomenon characteristic of the many nuclei that normally occur in an amoeba cell of the species used.^{6, 12} Synchronous nuclear division is also characteristic of the offspring of X-irradiated systems fused with nonirradiated amoebae.

When supralethally X-irradiated amoebae received enucleated light portions of nonirradiated centrifuged cells, their irradiated nuclei underwent mitosis

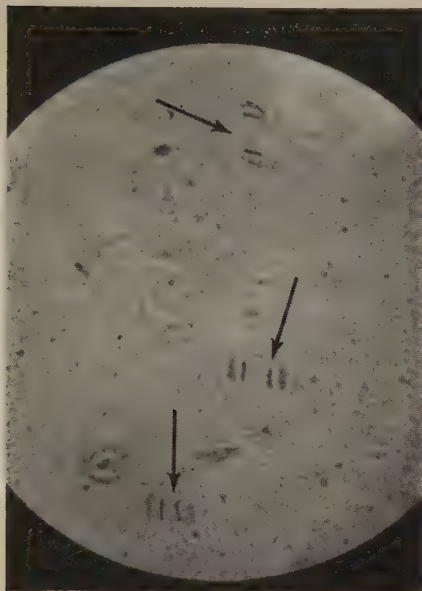


FIGURE 2. Three anaphase figures (arrows) of the first division in a supralethally X-irradiated amoeba that received nonirradiated cytoplasm from the light (centripetal) half of an amoeba centrifuged at 1400 g. $\times 500$.

(FIGURE 2) and these amoebae produced mass cultures. This shows that the nuclei in supralethally irradiated cells, which rarely divide once without the presence of nonirradiated protoplasm, can be induced to undergo repeated, apparently normal mitoses accompanied by cell division. The first cell divisions following the fusion of X-irradiated amoebae with either centrifuged or non-centrifuged portions of whole amoebae were frequently delayed, but later divisions were not.

Fission neutrons. Nonirradiated protoplasm from pelomyxae also prevents death in supralethally neutron-irradiated organisms. The data show that 91 per cent of 33 neutron-exposed amoebae survived after microfusion with half or third portions of nonirradiated cells.⁴ Recovery occurred in all cases in which the fused cell contained at least one part nonirradiated to about 15 parts irradiated protoplasm. This general volume relationship holds also for supralethally X-irradiated cells. The first postradiation cell division was delayed in these fused cells, but later divisions were not.

Ultraviolet radiation. Pelomyxae were given a supralethal dose of ultraviolet radiation ($2.5 \times$ minimum dose, which killed all of 50 exposed cells in 45 days) and then fused with nonirradiated amoebae, or with portions cut from them. A total of 21 fusions was made in which protoplasmic transfer was directly observed. Seventy-six per cent of these fused protoplasts survived and produced mass cultures. Evidence from stained specimens indicates that both irradiated and nonirradiated nuclei divided synchronously at the first mitosis following ultraviolet exposure and fusion. This is shown by the uniformly young nuclei

and absence of old nuclei in the daughter cells that were fixed only a few minutes after mitosis. Cell division was not significantly delayed in these fused organisms. The mean time and standard error of the first cell division after the isolation of 50 nonirradiated control cells was 3.0 ± 0.2 days; in the fused organisms (irradiated plus nonirradiated) the equivalent time was 3.7 ± 0.4 days. The mean time lapse between first and second divisions in the controls was 2.6 ± 0.1 days, while the equivalent time in the daughter cells of the fused organisms was 2.5 ± 0.3 days. Fusion alone does not delay division.⁷

Relative Restorative Value of Different Centrifuged Strata

It has been possible to obtain evidence on the survival and function of irradiated nuclei in a cell that contains both irradiated and nonirradiated whole protoplasm or irradiated whole protoplasm and nonirradiated cytoplasm (FIGURE 2). However, information on the function of smaller cytoplasmic components in similarly combined systems has been more difficult to obtain. It has not been possible, after extensive cytological examination and phase microscopic study, to see morphologic changes in the smaller components, at least until some time after radiation exposure. Consequently, the nonirradiated donor cells were centrifuged so that certain of the contents in a given amoeba could be separated and concentrated into strata within the living system. These strata were then isolated by cutting the cell into segments, each highly enriched with certain components. None of the components in any of the segments, however, was uncontaminated by other intracellular materials, as Holter has pointed out.¹³⁻¹⁵ These segments or portions of amoebae were then injected by microfusion into different irradiated cells, which were then isolated from other amoebae and cultured.

Preliminary work on the therapeutic value of different centrifuged strata has been done on X-irradiated cells.³ More recent data on experiments with supraethally X-irradiated as well as neutron-irradiated pelomyxae have added to this information.^{4, 11} Gravitational forces were varied, while the time was kept constant at 10 min. Regardless of the gravitational force used, the heavy portions of nonirradiated amoebae prevented death in about 90 per cent of the supraethally X-irradiated or neutron-irradiated recipients. Most of the resultant cells underwent cell division and produced mass cultures. When the centrifuged cells were cut into thirds, the middle portions of stratified cells were also of therapeutic value equivalent to that of nonirradiated, noncentrifuged systems. The survival values obtained after the fusion of the X-irradiated cells with heavy halves agree with those of neutron-irradiated cells that received middle and heavy portions.

The light half and third portions progressively lost their therapeutic capacity as the centrifugal force was increased. FIGURE 3 shows this relationship graphically for X-irradiated cells that received light halves of nonirradiated cells. A similar curve was obtained following the fusion of supraethally neutron-irradiated amoebae with nonirradiated light thirds, indicating that an increasingly higher percentage of light halves lost their therapeutic capacity as the centrifugal force was increased. Presumably, the reason for this lies in

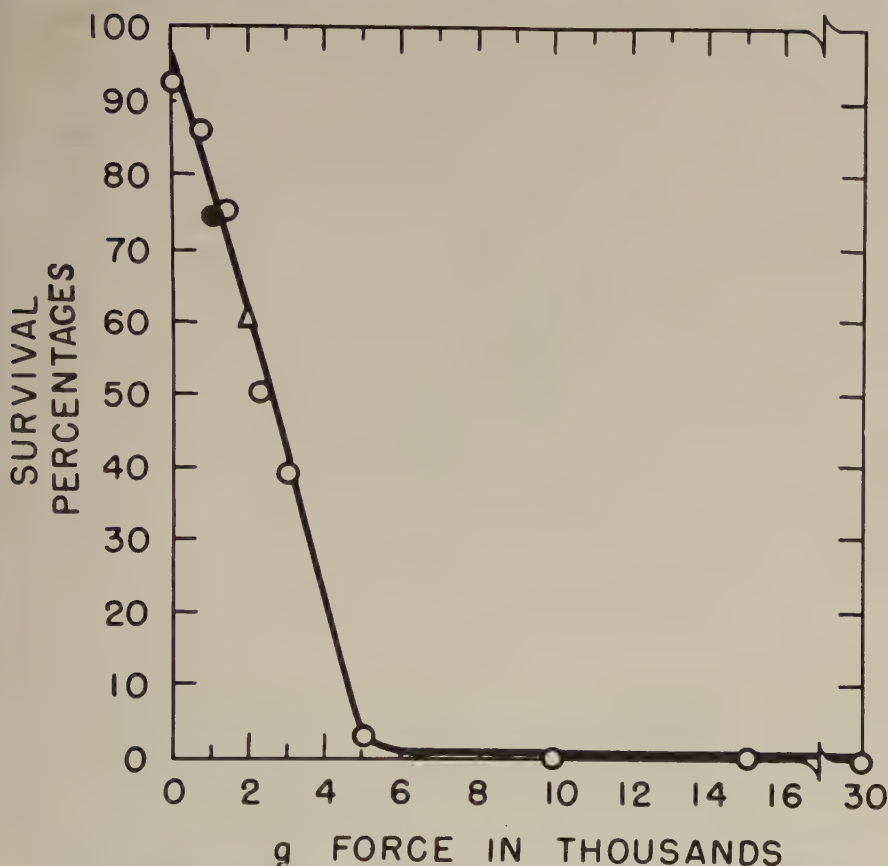


FIGURE 3. The relation between gravitational force and capacity of light portions of non-irradiated, centrifuged amoebae to prevent death. The solid circle (●) and open triangle (Δ) are based upon later experiments of 20 and 28 fused amoebae, respectively. Data for the open circle (O) points are available in reference number 11.

the loss of essential therapeutic components from the light portions and, if so, the heavy half protoplasm should become more effective per unit volume as the light half protoplasm becomes less potent per unit volume. This appears to be the case according to experience with microfusion. However, further knowledge of the relative effectiveness per unit volume of different centrifuged portions depends upon more precise measurements on the volume of injected protoplasm, as well as on the volume of the recipient cell.

Migration of Intracellular Components During Centrifugation

Normally, the nuclei and other particulate components of *pelomyxa* cytoplasm appear to be randomly distributed (FIGURE 4). The cytological data pertaining to the migration of intracellular components is described in some detail elsewhere.¹¹ Suffice it to say here that following 700 g or more



FIGURE 4. The giant multinucleated amoeba, *Pelomyxa illinoisensis*. ($500 \times 200 \mu$). Delafield's hematoxylin stain. N = nuclei.

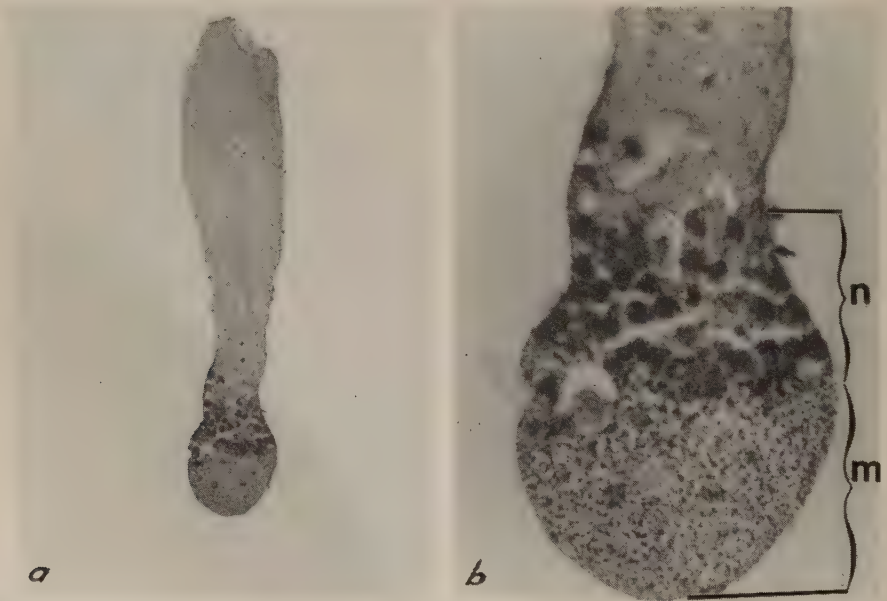


FIGURE 5. (a) Low ($\times 107$) and (b) high ($\times 355$) magnifications of a longitudinal section of an amoeba centrifuged at 715 g for 10 min. Altman's aniline acid-fuchsin stain. N = nuclear stratum. M = stratum of mitochondria. Heavy pole toward bottom of page.

the nuclei most mitochondria, food vacuoles, most crystal-like bodies, heavy spherical bodies,¹⁶ and the dense optically clear cytoplasm migrate to the centrifugal pole and, consequently, are found in the heavy halves and thirds of cut cells (FIGURES 5a and b). The middle thirds often contain some nuclei and mitochondria, but the light halves and thirds are cleared of nuclei, and essentially cleared of mitochondria, after 700 g for a 10 min. period. The light thirds contain fat globules, fluid and crystal vacuoles, and the less dense hyaline cytoplasm. The fine particulate components (0.1 to 0.3 μ) migrate centrifugally, predominantly between 1000 and 7000 g, according to phase microscopic observations on living halves and thirds of centrifuged cells.

Fusion of Differently Irradiated Cellular Systems

As would be expected, the fusion of one supralethally X-irradiated amoeba with another does not prevent death or extend the time of death, as shown by 42 fusions that were made combining pairs of amoebae each of which had been given a lethal dose of X rays.¹¹ Theoretically, the therapeutic component(s) in nonirradiated protoplasm that prevent death in supralethally X-irradiated amoebae might not be damaged in cells given supralethal doses of certain other types of radiation. This concept was directly tested for two radiations other than X rays.

Supralethally neutron-irradiated amoebae were combined with portions cut from supralethally X-irradiated amoebae,⁴ so that the fused protoplasts each contained both types of irradiated protoplasm. The average ratio of X-irradiated to neutron-irradiated protoplasm was approximately 1 to 8. The data from a total of 21 fusions show clearly that these systems are not mutually helpful, and survival does not follow this type of union.

As an extension of this idea, supralethally X-irradiated cells were fused with supralethally ultraviolet-irradiated amoebae. A total of 21 fusions was made, and the protoplasts, each containing X-irradiated and ultraviolet-irradiated protoplasm, were isolated and fed. All of these organisms died. Most of them died between the fourth and sixth days after exposure, and only one divided. In the latter case only a single division occurred and both daughter cells died.

DISCUSSION AND CONCLUSION

If a lethal dose of radiation or other toxic agent critically damages only certain kinds of intracellular components essential to the life of the cell, it should be possible specifically to replace or repair the damaged units. In using this hypothesis, a major problem is to find which units are critically damaged by radiation and to discover a means of helping the cell to overcome the damage. This can be done by introducing a whole new spectrum of ingredients necessary to a living cell, namely, whole protoplasm from the same species. The major knowledge that this approach has given thus far, however, is that radiation death can be prevented by the injection of whole protoplasm, and that irradiated and nonirradiated nuclei can continue to undergo mitosis in an apparently normal manner in the same cell.

Experience with the fusion of lethally irradiated amoebae with different strata of centrifuged, nonirradiated amoebae has shown principally that certain segments of stratified cells can also prevent radiation death following fusion, and that the presence of nonirradiated nuclei is not essential for recovery. Similarly, the nonirradiated mitochondria do not appear to be required. It is clear that the following components from nonirradiated cells are also definitely not required for recovery: fat globules; contractile and other optically clear vacuoles of various sizes; fresh food vacuoles; small crystal vacuoles that migrate centripetally; and the optically clear cytoplasm of the light portions. Evidence appears to show that the cytoplasmic component(s) that are capable of preventing radiation death are those that migrate centrifugally more slowly than the following: heavy spherical bodies; crystal-like bodies; food vacuoles; mitochondria; nuclei; and the most dense optically clear cytoplasm. This leaves the particulates finer in size than acid-fuchsin staining mitochondria, and/or the optically clear cytoplasm that suspends them, as the elements seemingly capable of preventing radiation death. According to Holter,¹⁴ these fine granules ($0.25\ \mu$) are the morphologic correlates of mammalian microsomes. However, these fine granules in *P. illinoisensis* have not been studied biochemically. Although quite uniform in optical appearance,⁶ it is possible that some of them differ in biochemical activity.

If one type of radiation (X rays) does not incapacitate the same vital components in a cell that another type of radiation (fission-neutrons or ultraviolet) does, then two of these protoplasmic systems might be mutually beneficial following fusion. This is of theoretical interest particularly since the initial physical action of these radiations is different. X rays bring about ionization by the production of recoil electrons, whereas fast neutrons produce it by recoil protons. In addition to this, the ionization tracks are different. On the other hand, ultraviolet radiation causes excitation and is differentially absorbed at specific wave lengths. At the wave length used ($2537\ \text{\AA}$) the nucleic acids absorb heavily.¹⁷ However, as shown in this article, there were no mutually beneficial effects of cells exposed to different radiations and then combined in the manner described. This indicates that fission neutrons as well as ultraviolet photons incapacitate the therapeutic component required for survival in supraethally X-irradiated amoebae. As far as this therapeutic aspect of radiation injury is concerned, these three different radiations appear to act either on the same substances or on different substances localized on the same organelle. In other words, each of these radiations probably affects a number of different intracellular units, but the protective component(s) of special interest in the present work seem to be directly or indirectly damaged by each type of radiation that was used. If the fine particulates are the target organelles, several roads to their common inactivation could be traveled: energy dissipation at any one of several lower (biochemical) organizational levels might result in a useless fine particle.

The greatest delay in cell division among survivors, after various doses of ultraviolet radiation (FIGURE 1), occurred prior to the first division according to mean values of control and experimental groups. This delay was from two to three times that of the nonirradiated control. The mean time lapse between

the first and second cell division was delayed nearly as much, being about twice that of the corresponding nonirradiated controls. The third division means, however, showed only slight delay, and mass cultures of 20 or more cells were produced in the single cell clones within a few days after the third division occurred. Thus, the greatest delay occurs prior to the first and second cell divisions after irradiation, as is the case following sublethal exposure to ionizing radiations (X rays, gamma rays, and fission neutrons).

In *Paramecium* X rays as well as nitrogen mustard (HN2) cause delay in cell division that is greatest before the first division, and the delay gradually decreases between successive divisions,¹⁸ as in the case of *Pelomyxa carolinensis*⁸ and *P. illinoisensis*,¹ except that in the latter organism the greatest delay sometimes occurs after the first division but not later than this. It is interesting that in X-ray-treated *P. carolinensis* the block in cell division occurs before the first division, but following HN2 treatment the block occurs after the first division.¹⁹ If well fed, rapidly dividing paramecia are exposed to UV, they show a major cell division delay period after some 2 or 3 divisions, and this is followed by recovery in sublethally irradiated organisms.^{17, 20} However, less rapidly dividing or starved paramecia, if exposed to UV, might be expected to show the lag in cell division earlier, before the first division or immediately afterward. A special study of this nutritional aspect has not been made on *Pelomyxa*. Up to the present time very little use has been made of starved or poorly fed amoebae in this laboratory. Wilber and Slane²¹ exposed starved *P. carolinensis* amoebae to 2537 Å UV radiation, but the organisms were not fed again after irradiation. Mazia and Hirshfield²² have shown that well-fed *A. proteus*, which is at least 10 times more resistant to X rays than *P. illinoisensis*,^{1, 2} has an ultraviolet sensitivity almost identical to that of *P. illinoisensis*. Furthermore, cell division delay in UV-exposed *A. proteus*²² is essentially complete after one or two postirradiation divisions.

SUMMARY

The comparative effects of X rays, γ rays, fission neutrons, and ultraviolet radiation on the giant amoeba *P. illinoisensis* have been shown. Particular attention has been given to the percentages of survival and length of life after various exposures.

Major emphasis has been placed upon the therapeutic value of nonirradiated protoplasm and some of its centrifuged portions following a special method of microinjection known as fusion. Whole protoplasm, as well as heavy portions of centrifuged amoebae, prevent radiation sickness and death after injection into supralethally irradiated amoebae. On the other hand, light portions of nonirradiated, centrifuged amoebae progressively lose this capacity as the centrifugal force is increased. Nuclei are not necessary components of therapeutic portions.

Fission neutron as well as ultraviolet radiation bring about the inactivation of the therapeutic component in nonirradiated protoplasm that otherwise prevents death in supralethally X-irradiated amoebae. Implications of these findings are discussed.

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References

1. DANIELS, E. W. 1955. X-irradiation of the giant amoeba, *Pelomyxa illinoensis*. I. Survival and cell division following exposure. Therapeutic effects of whole protoplasm. J. Exptl. Zool. **130**: 183-197.
2. ORD, M. J. & J. F. DANIELLI. 1956. The site of damage in amoebae exposed to X-rays. Quart. J. Microscop. Sci. **97**: 29-37.
3. DANIELS, E. W. 1956. Recovery from lethal X-radiation injury in amoebae after injection of centrifugal portions of nonirradiated cells. Radiation Research. **5**: 604-605.
4. DANIELS, E. W. & H. H. VOGEL, JR. 1958. Recovery following injection of nonirradiated protoplasm into amoebae irradiated with fission neutrons. II. Protective effect of centrifuged cell portions. In Semiann. Rept. Biol. and Med. Research Div. Argonne Natl. Lab. ANL-5841: 139-143.
5. KUDO, R. R. 1950. A species of *Pelomyxa* from Illinois. Trans. Am. Microscop. Soc. **69**: 368-370.
6. KUDO, R. R. 1951. Observations on *Pelomyxa illinoensis*. J. Morphol. **88**: 145-184.
7. DANIELS, E. W. 1954. Cell division in the giant amoeba, *Pelomyxa carolinensis*, following X-irradiation. II. Analysis of therapeutic effects after fusion with nonirradiated cell portions. J. Exptl. Zool. **127**: 427-462.
8. DANIELS, E. W. 1951. Studies on the effect of X-irradiation upon *Pelomyxa carolinensis* with special reference to nuclear division and plasmatomy. J. Exptl. Zool. **117**: 189-210.
9. PACE, D. M. & B. W. McCASHLAND. 1951. Effects of low concentrations of cyanide on growth and respiration in *Pyelomyxa carolinensis* Wilson. Proc. Soc. Exptl. Biol. Med. **76**: 165-168.
10. DANIELS, E. W. 1956. Relationship of cell division to survival in irradiated giant amoebae. Effects after an LD₇₀ dose of X-rays. Quart. Rept. Biol. and Med. Research Div. Argonne Natl. Lab. ANL-5518: 194-195.
11. DANIELS, E. W. 1958. X-irradiation of the giant amoeba, *Pelomyxa illinoensis*. II. Further studies on recovery following supralethal exposure. J. Exptl. Zool. **137**: 425-442.
12. MCCLELLAN, J. F. 1954. Observations on nuclear division in *Pelomyxa illinoensis*. Ph.D. Thesis. Univ. Ill. Urbana, Ill.
13. HOLTER, H. 1954. Enzymatic studies on mitochondria of amoebae. In Symposium on Fine Structure of Cells, Leiden. **8**: 71-76. Interscience. New York, N. Y.
14. HOLTER, H. 1954. Distribution of some enzymes in the cytoplasm of amoebae. Proc. Royal Soc. London. **B 142**: 140-146.
15. HOLTER, H. 1952. Localization of enzymes in cytoplasm. Advances in Enzymol. **13**: 1-18.
16. ANDRESEN, N. 1942. Cytoplasmic components in the amoeba *Chaos chaos* Linne. Compt. rend. trav. Lab. Carlsberg. Sér. chim. **24**: 139-184.
17. GIESE, A. C. 1953. Protozoa in photobiological research. Physiol. Zool. **26**: 1-22.
18. POWERS, E. L. 1955. Radiation effects in *Paramecium*. N. Y. Acad. Sci. **59**: (4) 619-636.
19. DANIELS, E. W. 1952. Some effects on cell division in *Pelomyxa carolinensis* following X-irradiation, treatment with bis(β -chloroethyl)-methyl amine and experimental plasmogamy (fusion). J. Exptl. Zool. **120**: 509-523.
20. KIMBALL, R. F. & N. GAITHER. 1951. Influence of light upon the action of ultraviolet on *Paramecium aurelia*. J. Cellular Comp. Physiol. **37**: 211-233.
21. WILBER, C. G. & G. M. SLANE. 1951. The effect of ultra violet light on the protoplasm in *Pelomyxa carolinensis*. Trans. Am. Microscop. Soc. **70**: 265-271.
22. MAZIA, D. & H. I. HIRSHFIELD. 1951. Nucleus-cytoplasm relationships in the action of ultraviolet radiation on *Amoeba proteus*. Exptl. Cell Research. **2**: 58-72.

THE CELL-TO-CELL TRANSFER OF NUCLEI IN AMOEBAE AND A COMPREHENSIVE CELL THEORY

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About eleven years ago I commenced a study of cellular inheritance by the method of transferring nuclei from one cell to another by micromanipulation. This technique was developed first by Comandon and de Fonbrune (1938). The reasons for undertaking this relatively arduous and protracted investigation were both general and particular: in general because, in certain respects, the study of inheritance by Mendelian methods can be misleading and because, in any case, Mendelian methods are at present useless for the study of somatic cells. In particular, this approach seemed essential for a proper understanding of the nature of the cellular changes that occur in carcinogenesis and in aging. The first results of this study were presented at the Congress of Cellular Biology held at Yale University, New Haven, Conn., in 1950 (Lorch and Danielli, 1950). The experimental work was successful with amoebae, although we had no success with ova. However, Briggs and King (1952) have been very successful in their studies with amphibian eggs, and there is no doubt that in the next decade our knowledge of the cytoplasmic aspect of cellular inheritance will be greatly extended.

The experimental work to be described in this paper will be concerned with the long-term results of transfers of nuclei between various strains of *Amoeba proteus* and *Amoeba discoides*. The short-term effects, the techniques, and the various essential control experiments have been described elsewhere (Lorch and Danielli, 1953a, b; Danielli, 1958). As in earlier publications, the following symbols have been used: *D* and *P* indicate the species *discoides* and *proteus* respectively, the suffixes *n* and *c* denote nucleus and cytoplasm respectively, whereas strains are indicated by prefixes. Thus, $\tau_1 P_n$, $\tau_1 D_c$ indicates an amoeba having a nucleus derived from *proteus* strain τ_1 and a cytoplasm from *discoides* strain τ . The strains mentioned here are $\tau_1 P$ and τD , obtained from Sister Monica Taylor (Glasgow, Scotland); $_D P$ obtained from J. A. Dawson (New York, N. Y.); $_Z P$, which was collected by C. C.-Andresen in Zealand, Denmark; and $\tau_4 P$, which originated from Sister Monica's Laboratory, but was kept in the Department of Zoology at Bristol, England, for many years before coming to us. The distinctions between species among the large uninucleate amoebae are at present of doubtful significance. For example, Sister Monica Taylor has distributed *A. proteus* to many European laboratories, from four of which we have obtained strains. We have grown these four strains under identical conditions, and find that the apparently stable differences between them are greater than those found between $\tau_2 P$ and τD .

The characters studied may be classed conveniently as morphological, physiological, and macromolecular.

Morphological Characters

Nuclear size. The simplest characteristic we have been able to study is the diameter of the nucleus. We have measured the largest diameter of the nu-

TABLE 1

VALUES OF AVERAGES OF THE MAXIMUM DIAMETERS OF NUCLEI IN VARIOUS CLONES AND HETEROTRANSFER COLNES

Type Diameter	$\tau_1 P_n, \tau_1 P_c$ 45	$\tau D_n, \tau_1 P_c$ 44	$\tau_2 P_n, \tau D_c$ 38.6	$\tau D_n, \tau D_c$ 38.2
Type Diameter	$\tau_2 P_n, \tau_2 P_c$ 38	$z P_n, \tau_2 P_c$ 40.3	$\tau_2 P_n, z P_c$ 41	$z P_n, z P_c$ 44.5

cleus in several heterotransfer clones and compared the averages of these diameters and also the distribution of diameters. Careful measurements have been made on two heterotransfers from $\tau_1 P$ and τD , and on two heterotransfers from $z P$ and $\tau_2 P$. The results are shown in TABLE 1.

The clones based on species $\tau_1 P$ and τD have been studied fairly thoroughly over a number of years. In a heterotransfer the nuclear size is very close to that characteristic of the cytoplasmic type. However, in repeated determinations the differences between heterotransfer and the corresponding cytoplasmic species have persisted, indicating a detectable influence of the nucleus also.

With the clones based on $\tau_2 P$ and $z P$, the influences of nucleus and of cytoplasm appear to be roughly equal in determining nuclear size.

Analogous conclusions follow from the study of the distribution of nuclear sizes in a clone. FIGURE 1 shows curves for $\tau_1 P$, τD , and the heterotransfer $\tau_1 P_n$, τD_c . Characteristically, clones of $\tau_1 P$ have a three-peaked curve, whereas τD has almost a single sharp peak. The heterotransfer very closely resembles the cytoplasmic species, having one very pronounced peak at the value characteristic of its cytoplasm. The reverse heterotransfer, τD_n , $\tau_1 P_c$, is also close to the cytoplasmic type, giving a curve with three peaks in approximately the positions typical of $\tau_1 P$.

The conclusion following from the study of nuclear sizes is that the cytoplasm is just as important as the nucleus in determining nuclear size, and may be more important than the nucleus. There is no discernible tendency to approximate to a size characteristic of the nucleus, even after eight years.

Shape when moving. The shape assumed by an amoeba when moving, under standard conditions, changes continuously. However, when outline drawings are prepared of many individuals from clones of different species, noteworthy differences become evident. Characteristically, *A. proteus* (strain $\tau_1 P$) tends to form few but large pseudopods with smooth outlines, whereas *discoides* (strain τD) may form many pseudopodia, is often relatively sheetlike rather than cylindrical, and tends to have a serrated outline. A given outline drawing can thus be classified as typical (for example, *P* or *D*) or alternatively more like one strain than the other (that is, *1P* or *1D*). FIGURE 2 shows the results of a study of 3 clones, including the data for 1949 to 1954. Two points emerge clearly: for the parent strains *P* and *D* the curves are unequivocally different; and the heterotransfer, $\tau_1 P_n$, τP_c has a shape intermediate between those of the two parent clones. FIGURE 3 shows the data obtained in 1958 on these clones, and on a fourth heterotransfer clone of composition τD_n , $\tau_1 P_c$. This

fourth clone is also intermediate in character. It will be noted that each of the two heterotransfers, when examined by the present criterion, is somewhat closer to the cytoplasmic than to the nuclear species. The marked influence of the cytoplasm appears to stabilize within a few divisions after heterotransfer and then remain substantially unaltered. With the culture $\tau_2 P_n$, $\tau D_c(1)$ this has been the case for more than 8 years of vegetative reproduction at a near-optimal rate.

Shape in antiserum. The shape of an amoeba when moving must be the resultant of the action of many factors, a resultant of a complex interplay of forces and structures. When placed in a sufficient concentration of a specific antiserum, amoebae cease to move and assume shapes that are characteristic of the strain and that may differ profoundly from those assumed by the organisms when moving. The shapes assumed in antiserum must be independent of dynamic forces, and therefore more strongly influenced by morphological factors. Two comparisons of parent clones and heterotransfer clones are shown in FIGURE 4, the two heterotransfer clones being $\tau_2 P_n$, τD_c . In both cases the heterotransfer shape is clearly intermediate between the two-parent clones

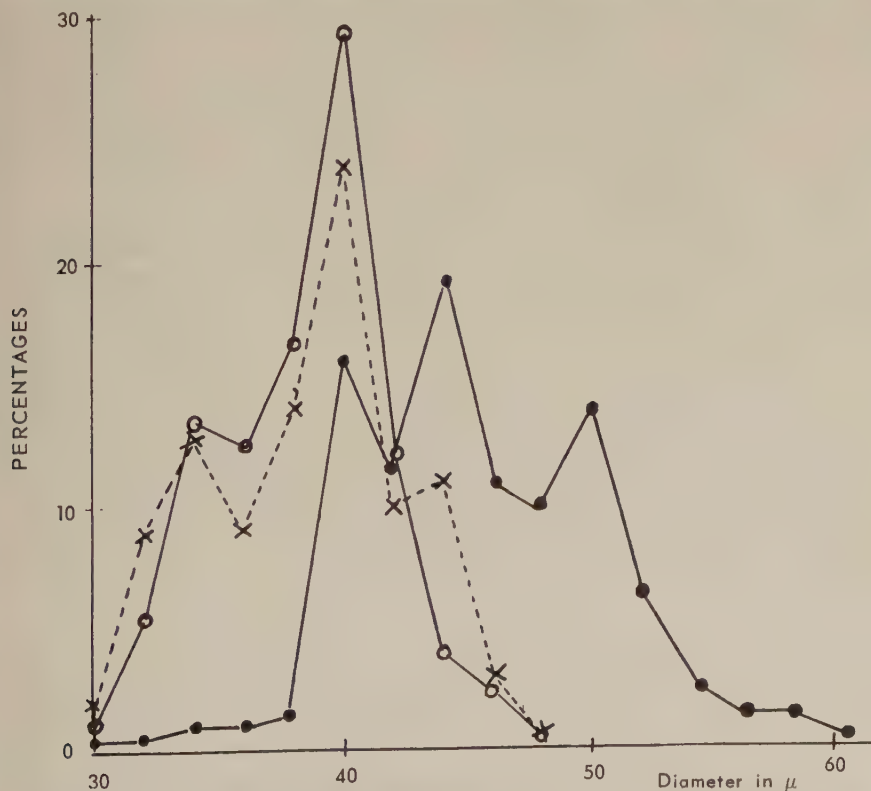


FIGURE 1. Distribution of nuclear diameters in $\tau_1 P$ (●), τD (○) and the heterotransfer $\tau_1 P_n$, $\tau D_c(1)$.

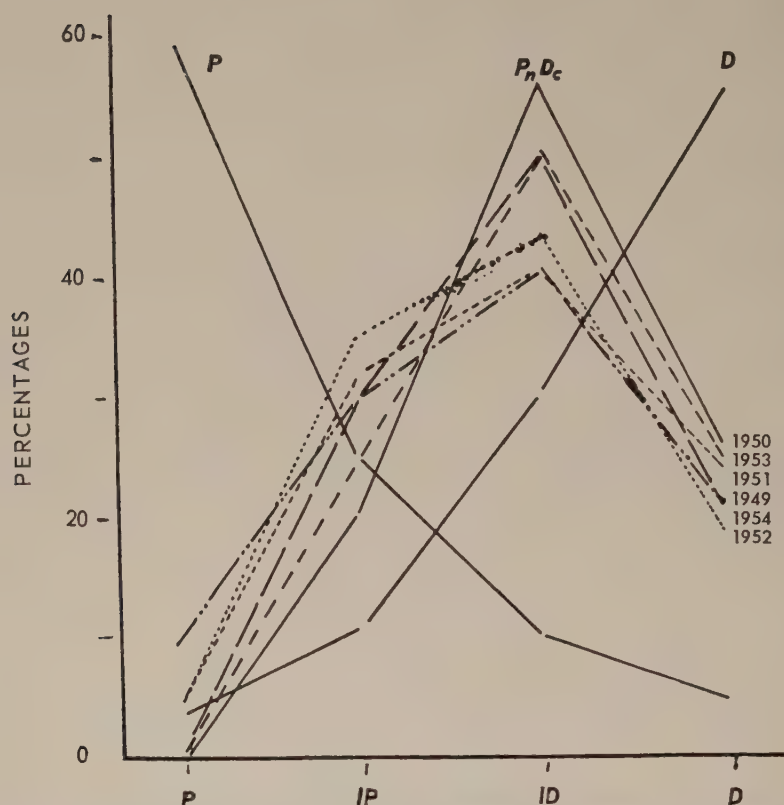


FIGURE 2. Distribution of shapes in clones of *A. proteus*, *A. discoides*, and a heterotransfer T_1P_n, TD_c (1).

From these and from all other morphological studies we have made to date, it is clear that both nucleus and cytoplasm have a powerful influence in determination of morphology, once a clone has become well-established. Furthermore, of the two, if either tends to exercise the larger influence, it is the cytoplasm that predominates. Once a clone has begun to multiply freely, there is no evidence that the cytoplasmic determinants diminish in their influence, even over a period as long as eight years.

Physiological Characters

Adaptation to antiserum. When subjected to a nonlethal concentration of a specific antiserum, some strains of amoebae become resistant to subsequent action of the same antiserum; others do not, or become resistant only to a minor degree. In TABLE 2 the data are set out for a number of strains and heterotransfers. For the limited number of examples available, it appears that the capacity to become resistant is carried exclusively by the nucleus. This may, in fact, be true, but it is equally possible that there are also cyto-

plasmic determinants involved that are carried by both the types of cytoplasm tested to date.

Rate of multiplication of clones. If the logarithm of the number of animals in a clone is plotted against time (normally measured in days, for this purpose) a straight line is obtained. If θ is the angle that this line makes with the time axis, $\tan \theta$ is an appropriate measure of the rate of growth. When different strains and species are examined, whether the growth rate follows the nuclear or cytoplasmic parent not only varies from one type of heterotransfer to another, but also with variation in experimental conditions. The chief data available are shown in TABLE 3.

For the heterotransfers derived from $\tau_2\dot{P}$ and τD , the rate of multiplication

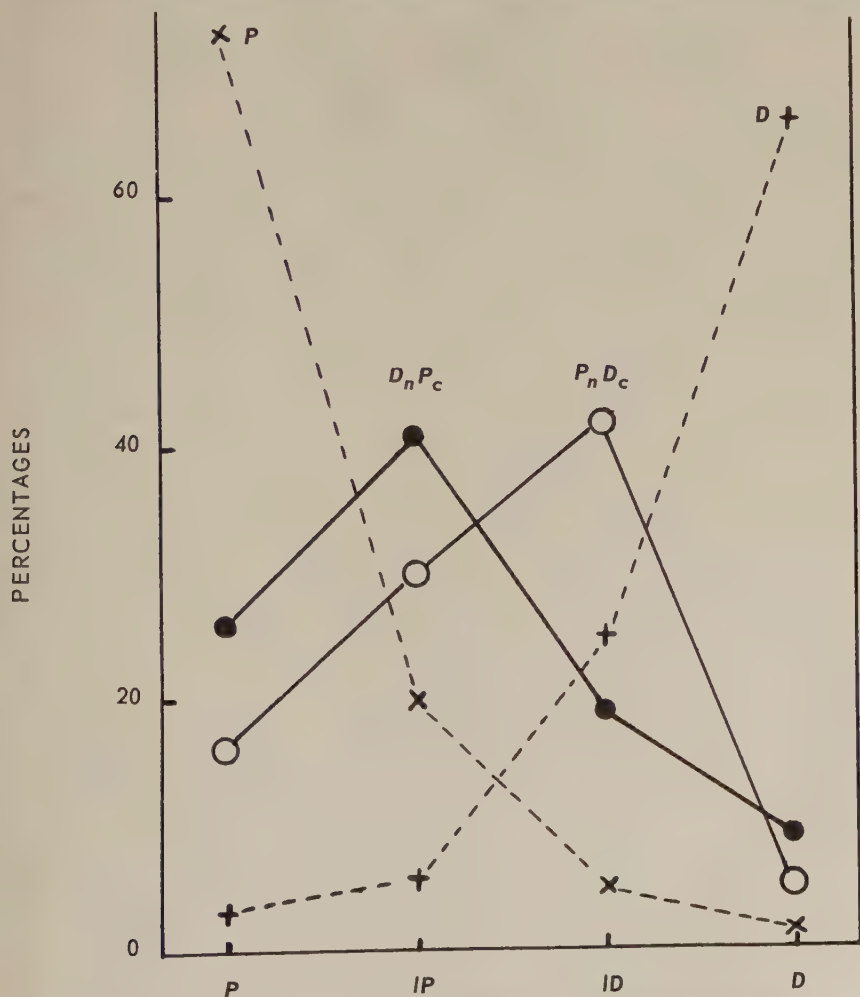


FIGURE 3. Distribution of shapes in two parent clones and the reciprocal heterotransfers.

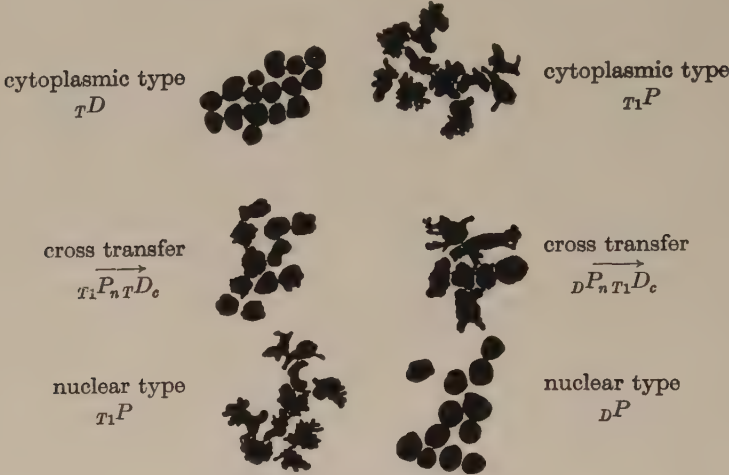


FIGURE 4. Silhouettes of amoebae immobilized by specific antisera.

TABLE 2

THE CAPACITY TO DEVELOP RESISTANCE TO SPECIFIC ANTISERUM IN CLONES OF, τD , $D P$, $\tau_1 P$ AND THEIR HETEROTRANSFER DERIVATIVE CLONES
Strains Capable of Developing Marked Resistance Marked +

<div>Nuclear type Cytoplasmic type</div>	$\tau_1 P$	$D P$	τD
$\tau_1 P$	—	—	+
$D P$?	—	?
τD	—	—	+

normally follows the values for the cytoplasmic species. However, after treatment of a heterotransfer with antiserum the rate is altered with the clone $\tau_2 P_n$, τD_c , slowing down to a value corresponding to the nuclear species. The reverse heterotransfer, τD_n , $\tau_1 P_c$, is not so much affected, although the rate of multiplication is increased and comes closer to the value for the nuclear species.

With the heterotransfers derived from $z P$ and $\tau_1 P$, the effect of temperature variation on rate of multiplication has been studied. The two parent clones, although morphologically very different, multiply at about the same rate at 17°C ., but at considerably different rates at 26° and at 11.5°C . The heterotransfers (TABLE 3) follow the cytoplasmic species at 11.5° , and the nuclear species at 26°C . The situation is obviously complex. Confirmation of this is obtained when the data for a given strain at different temperatures are compared. The temperature coefficient of growth is quite high, so presumably we are dealing with an activated process, and can write (assuming there is a single rate-limiting factor),

$$\text{rate of growth} = \tan \theta = \text{const.} \times \exp. (-\Delta F^*/RT),$$

TABLE 3
MULTIPLICATION RATES OF CLONES EXPRESSED AS $\tan \theta^*$ FOR TWO SETS OF
HETEROTRANSFERS AT 17° C.

Type	$\tau_1 P_n, \tau_1 P_c$	$\tau_1 P_n, \tau D_c$	$\tau D_n, \tau_1 P_c$	$\tau D_n, \tau D_c$
Tan θ (normal)	1.8	2.3	1.8	2.2
Tan θ after antiserum	1.9	1.7	1.9	2.8
Type	$\tau_4 P_n, \tau_4 P_c$	$\tau_4 P_n, z P_c$	$z P_n, \tau_4 P_c$	$z P_n, z P_c$
Tan θ at				
11.5° C.	0.35	0.25	0.42	0.23
17° C.	0.97	1.0	0.89	0.92
26° C.	1.9	2.4	1.5	1.5

* See text.

OR

$$\log \tan \theta = -\Delta F^*/RT \times \text{constant.}$$

where ΔF^* is the free energy of activation, R = gas constant, and T = absolute temperature. Thus, if the rate of growth of a clone depended on a single rate-determining process, $\log \tan \theta$ should be a linear function of $1/T$. This is not true; consequently, more than one rate-determining step is involved. There is nothing surprising about this, for a multiplication rate must inevitably involve a sequence of complex steps. For example, the rate may be determined by the rate of such factors as phagocytosis, of digestion, of the syntheses necessary for growth or of the mitotic process. Any one step may be rate-determining under appropriate conditions and, consequently, a much more detailed analysis of the control of multiplication rate would be profitable. All that can be said at the moment is that under some conditions cytoplasmic determinants, and under other conditions nuclear determinants, predominate.

Back-transfers. By transferring nuclei from a heterotransfer back to cytoplasm of their original species and by transferring nuclei of the original cytoplasmic species into the heterotransfer cytoplasm, it is possible to obtain a certain amount of information about changes that have taken place in a heterotransfer as a result of the symbiosis of nucleus and cytoplasm. Tests of this type have been made with two heterotransfers, $\tau_2 P_n, \tau D_c(1)$ and $\tau_4 P_n, z P_c(1)$. The evidence thus far is that both nucleus and cytoplasm change, although not rapidly, and that they become relatively incompatible with cytoplasm and nuclei of the parent strains. In the case of the first heterotransfer mentioned, marked incompatibility of both nucleus and cytoplasm with the original clones was relatively slow to develop (over several years) whereas, with the second heterotransfer, incompatibility was very marked a few months after the clone was established. A much more detailed study of this change is desirable, and also a study of how far it is reversible. However, one important conclusion may already be drawn: it is not the cytoplasm alone, or the nucleus alone, that changes. There is a drift from the properties of both the parent clones, not a drift toward either one or the other. Thus, in the interaction of determinants, those of nuclear origin and of cytoplasmic origin are of equal importance.

Macromolecular Characteristics

Our studies thus far have been confined to antigenic properties. These have involved measurement of the toxicity of various strain-specific rabbit antisera and also interaction of antisera with homogenized amoebae in agar gels, using the Ouchtolony technique.

Each strain of amoebae we have studied thus far (τ_1P , τD , νD and certain of their heterotransfers) has been most sensitive to antisera prepared to itself, but the situation has been greatly complicated by the fact that there are complex cross-reactions. When studied by the Ouchtolony method, it is clear that there are many antigens common to all strains, and a few that are strain-specific. In strains that have become resistant to an antiserum, the strain-specific antigens have been lost, but the common antigens, observed by the Ouchtolony method, are unchanged.

However, although the situation is at present far from adequately explored, the effect of treatment with antisera is to emphasize the importance of nuclear determinants. At present there is no reason to challenge the conclusion, based on studies of other organisms, that the nucleus determines the range of macromolecules that *can* be synthesized by the cell, whereas the cytoplasm and the environment have some influence in determining which molecules of this range are actually present at a given time.

If, as seems reasonable to suppose, the major function of the nucleus is that outlined above, we are left with the problem of accounting for the fact that, in this work, cytoplasmic determinants of inheritance appear to be fully as important as nuclear determinants. The hypothesis upon which we are working is that the cytoplasm controls the way in which macromolecules are organized into functional units (Danielli *et al.*, 1955; Danielli, 1958). Regulation of this activity, clearly essential, is at present in no way provided for by genetic theory. Nevertheless, I must emphasize the fact that there are other hypotheses that may need investigation.

A Generalized Cell Theory

When considering the data presented here, it is evident that nucleus and cytoplasm are of equal importance in regulatory activity, and that they constitute a single functional entity. Problems of control mechanisms necessary in such a complex system are becoming more and more evident. This is also true when other of the more complex fields of cellular biology are considered, for example, in embryonic differentiation, in the changes that occur in aging, and in carcinogenesis. This has led me to propose that an attempt be made to formulate a generalized cell theory in which all problems of cellular control mechanisms will find a proper place. The rudiments of such a theory were discussed at two meetings last year,* and this seems an appropriate occasion to consider a more complete outline. TABLE 4 shows five major divisions that such a theory must involve, and certain subdivisions of each of the major

* At the meeting organized by the Editorial Board of the *Journal of Comparative Morphology* at Cambridge, England, and at the International Congress of Gerontology at Merano, Italy.

TABLE 4
NECESSARY SUBDIVISIONS OF A GENERAL CELL THEORY

-
- | | |
|----|---|
| A. | <i>Intracellular control processes</i> |
| | (1) Control of nature of macromolecules present |
| | (2) Control of amounts and proportions present |
| | (3) Control of specificity of macromolecular interaction |
| | (4) Control of organization into supermacromolecular units |
| B. | <i>Cell and environment interaction mechanisms</i> |
| | (5) Mechanisms for examining and reacting to environment |
| | (6) Mechanisms for intercellular communication |
| C. | <i>Replication mechanisms</i> |
| | (7) Mechanisms for replication of macromolecules |
| | (8) Mechanisms for replication of higher structures |
| | (9) Mechanisms for cell division |
| | (10) Mechanisms for gene distribution to daughter cells |
| D. | <i>Energy acquirement and conversion</i> |
| | (11) Mechanisms for acquiring free energy from environment |
| | (12) Mechanisms for catalysis and control of catalysis |
| | (13) Mechanisms for conversion of energy into diverse forms |
| | (14) Mechanisms for restricting and increasing molecular fluxes |
| E. | <i>Mechanisms for genetic change</i> |
| | (15) Mechanisms for gene recombination |
| | (16) Mechanisms for new gene acquirement |
-

divisions. On inspecting this TABLE it will be evident that, although we have fairly detailed information about some of the subdivisions, there are others of which we are wholly ignorant. Let us consider the four subdivisions in category A, which is concerned with intracellular control processes.

(1) *The control of the nature of the macromolecules present in a cell.* This has long been recognized as necessary. Work of the last two decades has given strong support to the hypothesis that the chromosome genes, probably the DNA, carry the information that is required before any macromolecule can be synthesized. There may be exceptions to this rule. For example: viruses containing RNA are reproduced in cells, and it is possible that specific antibodies cannot be formed unless the cells concerned have experience of the antigens involved; in both these instances, the foreign virus or antigen may also be contributing information required for synthesis of the macromolecule concerned. It is not clear how far synthesis of virus and antibody macromolecules is also DNA-dependent, that is to say, whether the synthesis of a virus or antibody macromolecule is dependent also upon the presence of a specific chromosome gene. To put the matter in another way, the extent to which the foreign macromolecule acts as a stimulus and the extent to which it contributes essential information not carried by the host cell DNA remain to be established.

It is also clear that the presence of the relevant chromosome gene, although a necessary precondition for macromolecule synthesis, is not always a sufficient condition: often permissive and controlling factors must also be contributed, for example, in the case of adaptive enzyme synthesis.

The evidence thus far obtained by study of amoeba antigens is completely compatible with the hypotheses outlined above. As is well known in the case of *Paramecium*, the antigens actually synthesized by an amoeba are limited to those for which the nucleus carries the relevant determinants and which are

appropriate to the state of the cytoplasm. Thus, on transferring a nucleus from one strain to cytoplasm of another, an antigen may appear in the resultant heterotransfer clone that was not present in either parent clone. It remains to be seen whether the mechanism of development of resistance to an antiserum depends upon a simple switch of antigen synthesis or whether the situation is more complicated.

(2) *Control of the amounts and proportions of the macromolecules present in a cell.* This problem is just as important as controlling the nature of the macromolecules present in a cell. However, it has received remarkably little attention. Hinshelwood (1957) has shown that, if the reactions involved in synthesis are interlinked in certain ways, then in the steady state the ratios in which the different molecular species are synthesized remain constant. However, although this may be one of the operative mechanisms, it can provide at most for those aspects of growth that are relatively continuous. However, some of the phenomena encountered are discontinuous, for example, the synthesis of DNA, which is normally limited to a particular part of the intermitotic period. Looked at from an engineer's point of view, the necessary control processes involved would appear to involve both feedback and threshold phenomena. There are also well-defined effects associated with the chromosome genes, for example, position effects, modulator genes, and polygene phenomena. Although these are well-defined at the genetic level, we have almost no knowledge of the physicochemical mechanisms involved. How far these control processes are nuclear and how far cytoplasmic it will be impossible to determine until this vast field has been at least partially explored.

(3) *Control of specificity of macromolecular interaction.* If 2 macromolecules are to have the potentiality of interacting in a specific manner, there are at least 3 conceivable mechanisms. First, there may be some degree of correspondence between parts of the surfaces of the 2 macromolecules, as in antigen-antibody reactions. Second, there may be the possibility of binding the macromolecules together through the cementing action of smaller molecules, as in collagen. Third, a process from one macromolecule may fit into a recess or cavity reaching into the interior of the second molecule: examples of this are not known but one can, for instance, readily imagine that the carotenoid pigment of visual purple might bind the visual purple molecule into another molecule or structure.

The evidence available at present suggests that, insofar as potential specificity of interaction depends upon the properties of the macromolecules concerned, the ultimate control will be exercised by the chromosome genes. However, the supply of cement substances might be controlled equally well by the cytoplasm.

(4) *The control of organization of macromolecules into supermacromolecular units.* By supermacromolecular units I mean higher structures such as mitochondria, cell membranes, flagella, chloroplasts, centrosomes, and chromosomes. It is now well-known that the fact that a cell nucleus carries the information necessary for synthesis of the molecules required to build such a unit is not sufficient for that unit actually to be formed in a cell. Before such units can be formed, it seems necessary both that the nucleus should carry the necessary

information and that at least one of these structures should already be present in the cell. Consequently, the control of formation of higher structures in the cytoplasm rests in part in these structures themselves. This view is strongly supported by the studies of the morphological and physiological characteristics of amoebae reported here, in which the measure of cytoplasmic control has been striking.

The clarity of the evidence for cytoplasmic control of duplication of cytoplasmic structures throws into harsh relief two gaps in our knowledge: the way in which structures duplicate, and the origin of new structures in differentiation.

The first of these problems, the way in which structures such as chloroplasts and centrosomes duplicate, has been almost totally neglected. Some geneticists and biochemists even go so far as to claim that the problem does not exist and that, given the necessary molecules, such bodies will spontaneously form the correct structures. At present we do not know whether cytoplasmic structures duplicate by some form of "growth," followed by a type of binary fission, or whether some other principle is involved. Probably the most important step in this is to explore the problem by electron microscopy, after which the problem of control in the dimension of time may become more accessible.

The second problem, the origin of new structures in differentiation, is equally remote from solution in the terms which we are now using. The first aspect of this to be solved is whether the cytoplasm of an egg cell contributes determinants in a similar way, and in a similar degree, to that found in vegetative growth. The evidence obtained by fertilizing enucleated eggs of one species with sperm of another and also the results obtained by repeated back-crossing of hybrids with the paternal parent at first suggest that, in the last analysis, the cytoplasm contributes few or no determinants of a permanence equal to the chromosome genes, since the organisms so obtained usually approximate closely to the paternal species. However, there is also some evidence pointing in the opposite direction; two examples of this may be mentioned. First, there are some instances in which maternal effects persist through many generations where the nucleus contains no maternal genes. Second, hybrids and fertilized enucleated eggs do not develop unless they are made between closely related species: this "incompatibility" between unrelated sperm and egg cytoplasm must rest on some material basis. The simplest explanation of this incompatibility would be that the cytoplasm is not merely an organic matrix upon which the genes operate, but that egg cytoplasm, like that of differentiated cells, contributes essential, species-specific determinants. Thus, the relationship between nuclear determinants and cytoplasmic determinants in development remains to be settled.

To illustrate the value that even so simple an approach to a general cell theory as that outlined may have, let us note that, although points A1 to A4 are of equal relevance in the study of complex problems such as differentiation, aging, and carcinogenesis, points A2 and A4 have, so far, been largely ignored.

On homeostats. The foregoing discussion would be misleading if attention were not drawn to the fact that determinants of cellular behavior may exist that are neither cytoplasmic nor nuclear, but depend upon communication between

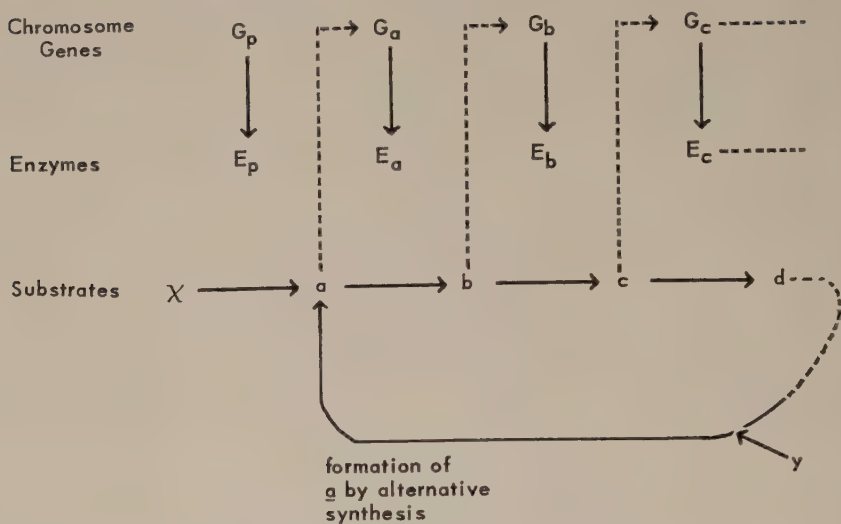


FIGURE 5. To illustrate the formation of a quasi-autonomous homeostat from a cyclic feedback system.

morphologically distinct cell organs. This is best illustrated by reference to FIGURE 5. The chromosome genes G_p , G_a , G_b . . . control formation of enzymes E_p , E_a , E_b . . . and the substance x is acted upon by this succession of enzymes to give a series of substrates a , b , c . . . , each of which is an essential stimulus for eliciting formation of such enzymes as E_a and E_b . Immediately we encounter a homeostatic process whereby, in the steady state, the amount of each enzyme present may be related to the amount of its substrate; what is much more interesting, however, is the fact that if the process becomes cyclic, so that a is formed by an alternative synthesis involving a substance y , the complex of genes and enzymes G_a , G_b , and E_a , E_b . . . is no longer dependent on the activity of gene G_p , but becomes a quasi-autonomous system. This system does not require any exact spatial relationship between the genes and their operative enzyme products, but only that there should be effective communication between them via the enzyme reaction products. Thus, it is quite feasible for the genes to be nuclear and the functional enzymes to be cytoplasmic. For example, the enzymes could be part of a mitochondrion, and a and y might be citric acid and acetyl-coenzyme A, respectively.

This type of gene-enzyme complex is self-perpetuating and homeostatic within certain limits. However, if any substrates such as a and b , are removed, for example, by active transport or by introduction of a second metabolic path, the complex becomes unstable, may disappear, and will not reappear unless a is again produced. It is possible that the alternative states of cytoplasm, in which different antigen sets are elicited from the same chromosome genotype, are in fact alternative complexes of this type. We can also see that analogous complexes may be responsible for the appearance and disappearance of individual cellular characteristics during differentiation.

Thus we observe, from two different points of view, that assemblages of

macromolecules may assert genetic effects other than those displayed by the chromosome genes. The first effect involves the provision of the information required if a set of macromolecules is to be assembled into a defined cell organ, and the second involves autonomous gene-enzyme complexes arising from feedback processes. I have suggested that such determinants should be called homeostats. A homeostat may be defined as "an organization of macromolecules, or of processes, which is self-reproducing and may also determine and control the expression of one or more characters in a cell lineage" (Danielli, 1956). The advantage of such a term is that it does not carry the emotional charge or associations involved with the term gene, and so makes it easier to think separately and independently of chromosome genes and homeostats.

Finally, it will be appropriate to comment on the characteristics that we may expect of different types of control processes. Feedback mechanisms can provide for the existence of alternative steady states and for oscillations of cellular activities; however, discontinuous processes, such as cell division and DNA synthesis, must depend on threshold phenomena, insofar as can be seen at present. The communication mechanisms between genes and enzymes, and between homeostats, are likely to depend in part upon simple relations between gene activations and enzyme substrates, as suggested by FIGURE 5, but there must also be more complex mechanisms. The active agents in the more complex systems are likely to be substances such as steroids and pharmacologically active substances such as acetylcholine and adrenaline. The likelihood is that these substances initially arose in cells as intracellular communication mechanisms and only later have acquired a value for intercellular communication.

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References

- BRIGGS, R. & T. J. KING. 1952. *Proc. Natl. Acad. Sci. Wash.* **38**: 41.
COMANDON, J. & P. DE FONBRUNE. 1938. *Ann. inst. Pasteur.* **60**: 113.
DANIELLI, J. F. 1956. *Nature.* **178**: 214.
DANIELLI, J. F. 1958. *Proc. Roy Soc. London.* **B148**: 321.
DANIELLI, J. F., I. J. LORCH, M. J. ORD & E. G. WILSON. 1955. *Nature.* **176**: 1114.
HINSHELWOOD, C. N. 1957. *Symposia Inst. Biol.* **6**: 1.
LORCH, I. J. & J. F. DANIELLI. 1950. *Nature.* **166**: 329.
LORCH, I. J. & J. F. DANIELLI. 1953a. *Quart. J. Microscop. Sci.* **94**: 461.
LORCH, I. J. & J. F. DANIELLI. 1953b. *Quart. J. Microscop. Sci.* **94**: 445.

CYTOPLASMIC DEPENDENCE IN AMOEBAE

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Many experiments performed in D. Mazia's laboratory, as well as in our own, indicate that cytoplasmic activity in amoebae is under very close nuclear control. In other cells, such as the unicellular alga *Acetabularia*, sea urchin or amphibian eggs, unripe red blood cells (reticulocytes), synthetic activities persist much longer than in amoebae: for instance, net protein synthesis occurs in anucleate fragments of *Acetabularia* (J. Brachet *et al.*¹ and G. Werz²), provided that light is supplied to the algae. There is no doubt that reticulocytes can incorporate amino acids very actively into their proteins (H. Borsook *et al.*,³ B. W. Holloway and S. H. Ripley,⁴ S. B. Koritz and H. Chantrenne;⁵ F. Gavosto and R. Rechenmann⁶) and it has even been claimed that these anucleate cells can synthesize enzymes (Koritz and Chantrenne⁵) and hemoglobin (A. N. Nizet and S. Lambert⁷). In newt eggs (H. Tiedemann and H. Tiedemann⁸) and in sea urchin eggs (H. M. Malkin⁹) removal of the nucleus exerts very little influence, if any, on protein and nucleic acid metabolism, as measured by the incorporation of labeled precursors into these large molecules.

As we shall see, enucleation in amoebae is invariably followed by a considerable decrease in anabolic activities. Why do amoebae behave differently from other cells? The answer to this question seems to be fairly simple: anucleate amoeba fragments very quickly lose their locomotion and pseudopodial activity; they thus become unable to catch the living prey on which they live. On the other hand, *Acetabularia* fragments retain an unimpaired photosynthetic activity (Brachet *et al.*¹), provided they are given light and CO₂. Under these conditions, their energy production can remain perfectly normal for a long time. Sea urchin and newt eggs are laden with foodstuff reserves (glycogen, fats, proteins), while reticulocytes presumably find in the blood stream the many materials they require. The main difference between amoebae and other cells, in their anabolic activities in the absence of the nucleus, very probably lies in the reduced energy supply in the anucleate cytoplasm.

For that very reason anucleate fragments of amoebae lend themselves very well to studies on the biochemical role of the cell nucleus. In these organisms the cytoplasm contains a relatively small endogenous or exogenous food supply; therefore, the effects of enucleation can be expected to be more dramatic than in other unicellular organisms.

In fact, impressive changes in shape and behavior occur very soon after the nucleus has been removed from an amoeba: within a few minutes the anucleate fragment becomes spherical and can no longer attach itself to the glass surface. The reasons for the contraction of anucleate fragments remain unknown. Interesting nuclear transfer experiments by J. Comandon and P. de Fonbrune¹⁰ have shown that introduction of a normal nucleus into the cytoplasm of an anucleate amoeba is followed by a very quick recovery of the locomotory (pseudopodia-formation) activity. If, however, the fragments have been

deprived of their nuclei for a few days, the graft of a fresh nucleus is no longer followed by a resumption of normal locomotory activity. Irreversible changes due to the lack of a nucleus have obviously occurred by that time. If one may venture a guess concerning the reasons for the rounding up of anucleate amoebae, it seems that ATP and phosphate metabolism may be involved. It is known that treatment with ATP, as well as enucleation, quickly results in contraction of the amoebae, which become spherical and cease forming pseudopodia (G. Kriszat¹¹). Furthermore, D. Mazia and H. Hirshfield¹² (confirmed by Brachet¹³) have shown that one of the earliest biochemical changes occurring after enucleation in amoebae is a strong decrease in the P^{32} uptake. It may well be that a membrane-localized ATPase (ecto-ATPase of T. V. Venkster and V. A. Engelhardt¹⁴) is involved in these early changes following enucleation. In fact, Engelhardt¹⁴ has demonstrated the presence of ecto-ATPase in nucleate red blood cells, but not in the adult mammalian erythrocytes, which lack a nucleus. Experiments presently proceeding in our laboratory have shown that intact amoebae have a weak but definite ecto-ATPase activity; however, it is not yet known whether this activity undergoes any changes after enucleation.

Whatever the outcome of these studies, there is no doubt that removal of the nucleus does not lead to any decrease in the total ATP content of the amoebae. Anucleate halves of amoebae have in fact a definitely higher ATP content than their nucleate counterparts, probably because they no longer use ATP in synthetic processes (Y. Skreb-Guilcher¹⁵). However, these anucleate halves display a considerably decreased capacity to maintain ATP in the phosphorylated condition in anaerobiosis (Brachet¹³). Apparently the biochemical mechanisms that permit anaerobic phosphorylation (presumably glycolysis) are less active in the absence of the nucleus. We shall soon see that such a conclusion is in keeping with studies concerning the effects of nucleus removal on carbohydrate metabolism in amoebae.

Oxygen consumption is also essentially normal in anucleate fragments of amoebae (Brachet¹³), a fact that disposes of the old theory of J. Loeb¹⁶ on the fundamental role of the nucleus in cellular oxidations. Taken together, the data obtained on ATP content and oxygen consumption strongly suggest that removal of the nucleus has little or no effect on oxidative phosphorylations; since it is well known that the mitochondria are the main site of oxidative phosphorylations, it seems likely that the nucleus exerts little control over the mitochondria themselves. Recent unpublished electron microscope studies made in collaboration with W. Bernhard and E. Baltus show, however, that removal of the nucleus easily produces a marked swelling of the mitochondria, but it is a well-known fact that swollen mitochondria are still capable of normal oxidative phosphorylations in homogenates.

While removal of the nucleus in an amoeba exerts no dramatic changes on oxygen consumption, it has definite effects on the qualitative nature of the substrates used (Brachet¹³). After 3 to 4 days the starving anucleate halves—in strong contrast to their nucleate counterparts—stop utilizing their carbohydrate and fat reserves. Instead, the total protein content markedly de-

creases, suggesting that proteolytic processes have become active and that protein, rather than glycogen and fats, is being utilized in the absence of the nucleus. Under the experimental conditions adopted in our laboratory, there is very little utilization of proteins in nucleate fragments during the first ten days of starvation.

The metabolic changes described thus far can easily be explained if one supposes that the diphosphopyridine nucleotide (DPN) co-enzyme of glycolysis and oxidations were synthesized in the amoeba nucleus. Such a hypothesis finds some support in the fact that, in liver, DPN synthesis is entirely a function of the nucleus, according to G. Hogeboom and W. C. Schneider.¹⁷ If DPN is synthesized in the nucleus, one would expect, of course, a decrease in the DPN content of the anucleate amoebae; as a result, glycogen utilization would stop and anaerobic glycolysis would become insufficient to keep ATP in its phosphorylated form under anaerobic conditions. Such a hypothesis would therefore explain two of the above-described findings. An additional but very plausible hypothesis should be added, however, in order to explain all the available results: namely, that only the soluble DPN would break down in the absence of the nucleus, while DPN which is bound to mitochondria, would not be affected. Experiments have been performed in order to test this hypothesis, but they have led, unfortunately, to conflicting results. While E. Baltus¹⁸ in our laboratory, found a marked decrease of the DPN content of amoebae after enucleation, A. I. Cohen¹⁹ who used different culture and assay techniques, observed no significant decrease in the DPN content after removal of the nucleus. Further experiments are sorely needed in order to solve this unfortunate contradiction. It might be worth mentioning, for future experimental approaches, that it is possible to increase 2 to 3 times the DPN content of amoebae when the nucleotide is added to the medium, together with a protein capable of inducing pinocytosis. Such an increased uptake of DPN exerts no visible effect on the locomotion and glycogen content of anucleate amoebae (E. Baltus, unpublished).

We already have seen that removal of the nucleus leads to an increased utilization of proteins. This fact raises an interesting question: do all proteins disappear at the same rate from the anucleate cytoplasm? The question can be answered by comparative studies on the enzymatic activities of nucleate and anucleate halves. Such studies have been made (Brachet¹³) and the outcome has been that, while a number of enzymes remain unaffected by removal of the nucleus (protease, enolase, amylase, ATPase), others show a decrease in activity. The drop is slow in the case of dipeptidase, which behaves very much as the total proteins, but is truly dramatic for esterase and acid phosphatase. Why the various enzymes studied behave differently remains unclear. It is tempting to suppose that the behavior of the different enzymes depends on their intracellular localization: mitochondrial enzymes may remain unaffected by enucleation; ubiquitous enzymes such as dipeptidase may follow the same trend as total proteins; and enzymes bound to the microsome may be especially nuclear-dependent. However, the recent enzymatic studies of H. Holter^{19, 20} suggest that the real situation in amoebae is probably more complicated than that.

Since the total protein content decreases more rapidly in anucleate than nucleate halves, it is to be expected that incorporation of labeled amino acids into proteins is smaller in the former than in the latter: this is what has been found by D. Mazia and D. M. Prescott²¹ and what we also have shown (J. Brachet and A. Ficq²²), using methionine and phenylalanine. In our own experiments with phenylalanine, the decrease in the incorporation into proteins was only 50 per cent up to 6 days after enucleation; by that time the incorporation mechanism was starting to break down, so that, after 10 days, incorporation in anucleated halves dropped to 25 per cent of the value found for nucleate halves. It is interesting to note that fasting alone exerts a marked inhibitory effect on the incorporation, even in the nucleate halves. After 10 days, they incorporated phenylalanine into the proteins at a rate only one third of what it was at the beginning of the experiment. Another point worth mentioning is that, as we shall soon see, the breakdown of amino-acid incorporation into anucleate halves coincides with a sudden and marked drop in the ribonucleic acid (RNA) content in these fragments.

In the experiments of Mazia and Prescott,²⁰ who used methionine and a counting technique, the dependence of the cytoplasm on the nucleus for incorporation into proteins was still more impressive. Both series of experiments, however, lead to the same general conclusion: removal of the nucleus quickly and markedly decreases protein anabolism in amoebae. On the other hand, the incorporation of amino acids into proteins is never entirely suppressed by enucleation. The nucleus, while exerting a strong control on cytoplasmic protein synthesis, cannot be considered as the exclusive center of protein synthesis in the cell.

A very similar story can be told in the case of RNA, except that cytoplasmic dependence upon the nucleus is stronger than for proteins: cytochemical observations, as well as quantitative analyses, have definitely shown that there is a steady and marked decrease in the RNA content of the anucleate cytoplasm (Brachet¹³). Mere fasting, under our experimental conditions, exerts little effect on the nucleate halves, which retain an almost normal RNA content even after 12 days; by that time the drop in the RNA content of the anucleate fragments is well over 60 per cent. It can be concluded that in the amoeba the nucleus exerts a very strong control on cytoplasmic RNA and, presumably, on the microsomal structures of which RNA is normally a major constituent. Recently attempts have been made, in collaboration with W. Bernhard and E. Baltus, to study the morphology of the basophilic material in the amoeba with the electron microscope; these attempts, unfortunately, have been frustrating because the amoeba lacks a well-defined endoplasmic reticulum or ergastoplasm. Small granules, resembling Palade's granules, can, however, be seen attached to vesicular membranes. However, the well-known heterogeneity of the cytoplasm in amoebae precludes any rigorous analysis; we can say only that the vesicular structure becomes much coarser in anucleate halves after a few days.

Our experimental data on the RNA content of nucleate and anucleate fragments of amoebae obviously are compatible with the view that cytoplasmic RNA originates from the nucleus. They do not necessarily prove such a

hypothesis, however, since the drop in the RNA content of the anucleate halves could be due to a number of other causes. The elegant nuclear transfer experiments of L. Goldstein and W. Plaut,²³ presented elsewhere in this monograph by W. Plaut, add strong support to the argument that RNA is really synthesized in the nucleus and transferred to the cytoplasm. However, as pointed out by Goldstein and Plaut²³ themselves, these experiments do not prove that the labeled material migrating from the nucleus to the cytoplasm is the RNA as it really existed in the nucleus: it may be a precursor of a more or less complex nature. Furthermore, proof that the RNA transfer can proceed only in one direction, that is, from the nucleus to the cytoplasm, is not as convincing as one would like: experiments in which a normal, "cold" nucleus is transferred into very strongly labeled cytoplasm have still to be performed.

However, taken together, the data presented by Goldstein and Plaut,²³ as well as our own,¹³ leave little doubt about the reality of a transfer of nuclear RNA in the cytoplasm. One question remains, however: is the complete synthesis of some RNA possible in anucleate cytoplasm?

Independent experiments by W. Plaut and R. C. Rustad²⁴ and by A. Ficq, Y. Skreb-Guilcher and J. Brachet have shown clearly that incorporation of adenine remains possible in the cytoplasm of amoebae long after the removal of the nucleus. Since the results obtained in our laboratory have not yet been published in detail, the experimental data are presented in TABLE 1.

The experiments show that fasting alone so strongly reduces the incorporation of adenine into RNA, even in nucleate halves, that the data become meaningless at eight days. There is no doubt, however, that removal of the nucleus quickly and strongly decreases the incorporation much more effectively than in the case of Brachet and Ficq's²² experiments with phenylalanine. However, as also found by Plaut and Rustad,²⁴ a nucleate cytoplasm always shows some activity: even when the actual RNA content of the cytoplasm is markedly decreasing, synthesis, turnover, or exchange reactions still proceed in the absence of the nucleus, but to a very limited extent.

In view of the disappointingly low uptake of labeled adenine into starving amoebae, we have recently studied the incorporation of labeled $C^{14}O_2$ into nucleate and anucleate fragments (R. Tencer and J. Brachet²⁵). This precursor is very well incorporated but it has, of course, the drawback of its low

TABLE 1
NUMBER OF TRACKS RECORDED BY AUTORADIOGRAPHY TECHNIQUE PER SURFACE UNIT IN FRAGMENTS TREATED FOR 6 HOURS IN STERILE CHALKLEY MEDIUM CONTAINING 4 μ c. OF C^{14} -LABELED ADENINE/ml.: AVERAGE VALUES FROM 3 INDEPENDENT EXPERIMENTS

	Days after enucleation		
	1	3	8
Nucleate	0.24	0.11	0.03
Anucleate	0.08	0.03	<0.01
Nucleate Anucleate ratio	3	3.7	>3

TABLE 2
AVERAGE NUMBER OF TRACKS PER SURFACE UNIT

	Days after enucleation		
	1	3	8
Nucleate	0.907	0.843	0.336
Anucleate	0.854	0.490	0.204
$\frac{\text{Nucleate}}{\text{Anucleate}}$ ratio	1.1	1.7	1.65

TABLE 3
AVERAGE NUMBER OF TRACKS PER SURFACE UNIT

	Days after enucleation					
	1		3		8	
	Proteins	RNA	Proteins	RNA	Proteins	RNA
Nucleate	0.525	0.382	0.510	0.333	0.230	0.106
Anucleate	0.611	0.243	0.342	0.148	0.165	0.039
$\frac{\text{Nucleate}}{\text{Anucleate}}$ ratio	0.85	1.55	1.5	2.2	1.4	2.7

specificity. The incorporation of $C^{14}O_2$ in the two types of fragments has been studied first with autoradiography methods after removal of the acid-soluble precursors. The main results, which have been presented thus far only in preliminary form, are summarized in TABLE 2.

These values correspond essentially to the incorporation into the combined proteins and nucleic acid fractions. It will be noticed that, while incorporation of $C^{14}O_2$ markedly decreases during fasting, removal of the nucleus has only a limited additional effect on the anabolic processes, even after eight days.

In TABLE 3, separate results are given for protein and RNA; the values for proteins are those found after ribonuclease digestion (RNase: 0.5 mg./ml.) on the slides. It should be pointed out that this procedure is probably neither quantitative nor entirely specific. We cannot exclude the possibility that RNase digestion removes not only RNA but also some soluble proteins; in fact, RNase digestion on the slides did not remove much more radioactivity than did water alone under the same experimental conditions (1 hour at $37^\circ C$).

Although no definite quantitative conclusion can be drawn from these cytochemical experiments, it is clear that the results are such as would be expected: the "RNA" fraction is under much closer nuclear control than is the "protein" fraction.

Biochemical studies on the incorporation of $C^{14}O_2$ in the RNA fraction have also been carried out by Tencer and Brachet;²⁵ here again, the results can be presented only as preliminary, since the RNA fraction is probably far from pure. In these experiments, as in those preceding, the fragments were treated

TABLE 4
SPECIFIC ACTIVITY OF THE "RNA" FRACTION*

	Days after enucleation			
	1	3	5	8
Nucleate	35	28	50	36
Anucleate	39	28	43	47
$\frac{\text{Nucleate}}{\text{Anucleate}}$ ratio	0.9	1	1.2	0.8

* In counts per minute per microgram.

TABLE 5
RADIOACTIVITY PER 400 FRAGMENTS IN THE RNA FRACTION

	Days after enucleation			
	1	3	5	8
Nucleate	24	13	41	19
Anucleate	23	13	17	7
$\frac{\text{Nucleate}}{\text{Anucleate}}$ ratio	1.05	1	2.4	2.7

for 7 hours with 56 μg . $\text{NaHC}^{14}\text{O}_3$ /ml. (4 μc ./ml.); after removal of the acid-soluble fraction with 0.1 N HCl and counting, the residue was extracted with 1 N HCl for 30 min. at 100° C. The U.V. absorption of this RNA-containing extract was measured; the results have been expressed either as the specific activity (TABLE 4) or as the radioactivity per fragment (TABLE 5).

The experiments should certainly be repeated under improved conditions for purification of the RNA fraction. However, since they gave consistent results, as shown by the comparison of TABLES 3 and 5, a few tentative conclusions may be drawn.

First, removal of the nucleus decreases synthetic processes in the cytoplasm; the effect is much greater in the case of RNA than in the case of proteins. Second, the specific activity of the RNA fraction remains essentially unchanged; the drop in the incorporation per fragment apparently reflects the decrease in the RNA content of the cytoplasm after removal of the nucleus rather than an inhibition of synthetic processes.

A third conclusion, which can be presented with greater confidence, is that complicated metabolic processes remain possible in the absence of the nucleus for a considerable time. There is no doubt that radioactive C^{14}O_2 ultimately becomes incorporated into RNA and proteins of the anucleate as well as the nucleate fragments.

Such a finding stands in good agreement with the above-presented results on the respiration and ATP content of amoebae halves.

In summary, our experiments lead to the general conclusion that the dependence of the cytoplasm upon the nucleus varies considerably according to the

biochemical processes under study; thus far it is in the case of RNA that the greatest degree of cytoplasmic dependence has been found, a fact in keeping with the probable nuclear origin of part (but not all) of the RNA.

One would of course like to know whether, in amoebae, nuclear and cytoplasmic RNAs are synthesized under a direct control of the genetic material, that is, the deoxyribonucleic acid (DNA) localized in the nucleus. Attempts have been made in collaboration with Ray Iverson to test experimentally this possibility by injecting crystalline deoxyribonuclease (DNase) in amoebae, but these attempts, unfortunately, have led only to negative results: the Feulgen reaction in the DNase-injected amoebae remained entirely normal, indicating that the enzyme had failed to reach or to digest intranuclear DNA. The problem of the DNA-RNA relationships, however, is of such fundamental importance that we intend to repeat these attempts with different DNase preparations. There is no doubt that amoebae are ideally suited for such experiments, as for so many others.

References

1. BRACHET, J., H. CHANTRENNE & F. VANDERHAEGHE. 1955. *Biochim. et Biophys. Acta.* **18**: 544.
2. WERZ, G. 1957. *Experientia.* **13**: 79.
3. BORSOOK, H., C. L. DEASY, A. J. HAAGEN-SMIT, O. KEIGHLEY & P. H. LOWY. 1952. *J. Biol. Chem.* **196**: 669.
4. HOLLOWAY, B. W. & S. H. RIPLEY. 1952. *J. Biol. Chem.* **196**: 695.
5. KORITZ, S. B. & H. CHANTRENNE. 1954. *Biochim. et Biophys. Acta.* **13**: 209.
6. GAVOSTO, F. & R. RECHENMANN. 1954. *Biochim. et Biophys. Acta.* **13**: 583.
7. NIZET, A. & S. LAMBERT. 1953. *Bull. soc. chim. biol.* **35**: 771.
8. TIEDEMANN, H. & H. TIEDEMANN. 1954. *Naturwiss.* **41**: 535.
9. MALKIN, H. M. 1954. *J. Cellular Comp. Physiol.* **32**: 481.
10. COMANDON, J. & P. DE FONBRUNE. 1939. *Compt. rend. soc. biol.* **130**: 740.
11. KRISZAT, G. 1949. *Arkiv. Zool.* **1**: 81.
12. MAZIA, D. & H. HIRSHFIELD. 1950. *Science.* **112**: 297.
13. BRACHET, J. 1955. *Biochim. et Biophys. Acta.* **18**: 247.
14. VENKSTERN, T. V. & V. A. ENGELHARDT. 1957. *Biokhimiya.* **22**: 813.
15. SKREB-GUILCHER, Y. 1955. *Biochim. et Biophys. Acta.* **17**: 599.
16. LOEB, J. 1899. *Wilhelm Roux' Arch. Entwicklungsmech. Organ.* **8**: 689.
17. HOGEBOOM, G. H. & W. C. SCHNEIDER. 1952. *J. Biol. Chem.* **197**: 611.
18. BALTUS, E. 1956. *Arch. intern. physiol. et biochim.* **64**: 124.
19. COHEN, A. I. 1956. *J. Biophys. Biochem. Cytol.* **2**: 15.
20. HOLTER, H. 1955. *In* Fine Structure of Cells. :71. Symposium 8th Congr. Cell. Biology. Leyden. Interscience. New York, N Y.
21. MAZIA, D. & D. M. PRESCOTT. 1955. *Biochim. et Biophys. Acta.* **17**: 23.
22. BRACHET, J. & A. FICO. 1956. *Arch. Biol.* **67**: 431.
23. GOLDSTEIN, L. & W. PLAUT. 1955. *Proc. Natl. Acad. Sci. U. S. A.* **41**: 874.
24. PLAUT, W. & R. C. RUSTAD. 1957. *J. Biophys. Biochem. Cytol.* **3**: 625.
25. TENCER, R. & J. BRACHET. 1958. *Arch. intern. physiol. biochim.* **66**: 443.

RESEARCH ON THE AMOEBA IN 2158 A. D.*

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This monograph has comprehensively summarized the active research done on the amoebae during the past two decades. Behind this is a history of the amoeba itself which apparently starts in 1755 when Roesel von Rosenhof described *der kleine Proteus*. In all probability, the organism described by Rosenhof was similar, if not identical, to the so-called giant amoebae presumably belonging to the genus *Pelomyxa*. There is no question that the amoebae studied by Rosenhof were large, since he mentioned holding the amoeba with a feather. Rosenhof also stated that the amoeba had a delicate outer "skin" that held together the granular contents and when the skin was torn, the amoeba disintegrated. This information, cited by Schaeffer,¹ was reported more than 200 years ago.

In this publication, and in the rest of the literature, one finds frequent mention of the micrurgical approach. The first micromanipulator was introduced by Schmidt in 1859, and much of the earlier work by M. A. Barber, G. L. Kite, R. Chambers, T. Peterfi, and P. de Fonbrune was done on the amoeba. Barber² described the enucleation of the amoeba; his procedure was essentially the same as that used by my students today. At best, the micrurgical techniques as we know them today are less than half a century old.

Many of the other techniques discussed in this monograph are more recent—so that, despite the 200 years of history of the amoeba behind us, the era of major achievements in the study of the amoeba probably goes back no further than the past 30 or 40 years.

Suppose we were planning a conference on the biology of the amoeba, 200 years hence—what would be the major topics? What should we expect to learn from the amoeba by 2158 that we have not already learned? Accordingly, with not too bright a crystal ball, I should like to project into the future—imagining that such a conference is being planned.

I should probably build the conference around five major areas of investigation. These areas are based on the anticipated progress and extrapolation. I should predict steady progress on the basis of current results and also on outstanding improvements in methodology. Thus, improvements in observing cellular structures, especially in living cells, better ways of performing certain operations and experiments, and better ways of processing data are of foremost consideration.

Perhaps this all boils down to the philosophy expressed by Mazia, who discussed the problems and difficulties of studying the life history of cells, especially if the aims are directed toward a search for mechanisms, albeit difficult and perhaps pretentious. However, Mazia goes on to say: "If one cannot search for mechanisms, he can at least seek for simplifications and generalizations, for the rules or laws governing the life history of the cell.

* A dinner address delivered March 24, 1958.

The object of such an attitude is to seek those facts that will increase the comprehensibility and reduce the mystery of the processes whereby the cell moves through Time. I contend, moreover, that this can be accomplished by the simple procedure of asking the cell questions about its life history and by avoiding the intrusion of our own notions as to how the cell ought to perform. Such an experimental philosophy—for to ask a question can only mean to design an experiment—has at least this merit: that if the cell refuses to answer the question, the chances are that it was not an intelligent question.”

To this, I must add that even intelligent questions asked of the cell may be unanswered. The reasons are not that the cell fails to respond, but rather that we cannot understand the answer nor do we suspect that an answer is being offered. In other words, the lines of communication between the cell and the investigator have not been properly established.

To this end, I should like to add some of my ideas on how questions may be more intelligently asked of the cell. It is probably understandable that my approach to the cell is micrurgical. Moreover, I use the term cell with the anticipation that, basically, all the procedures can be applied to the amoeba. The issues here are mainly of dimensions rather than of semantics.

Several years ago, Jack Harris and I⁴ developed a volumetric submicromanipulator with which known volumes of material could be removed from a cell and transplanted into other cells.⁵ Such transfer of subcellular substances is facilitated by siliconizing micropipettes so that the adherence of protoplasmic constituents to glass is minimized.⁶ These methods have been specifically applied to the transplantation of nucleoli⁵ and nucleolar inclusions, as may be found in cells of the renal adenocarcinoma of the frog. The relative dimensions of such structures, together with the micropipettes required, can be readily adapted toward similar studies on the amoeba.

For the transplantation of subcellular particles, it is wise to use micromanipulators with greater flexibility and more convenient means of positioning microneedles and micropipettes than is possible with the older types of screwed micromanipulators, including the volumetric submicromanipulator. The joy-stick controlled instrument, made by E. Leitz, is one of the more recent and satisfactory innovations.

In 1926, as an undergraduate student at the University of Nebraska, I built my first micromanipulator.⁷ Since then nearly all my work has dealt with micrurgical instrumentation on the one hand, and with applications to cellular biology on the other. With more than 30 years of experience, it has frequently occurred to me that there should be an easier way of doing even the most difficult micrurgical operations. With this attitude and with the good fortune of having Jack Harris, an expert engineer and instrument maker as an associate, my thoughts quite naturally have drifted toward automation. Actually, an attempt to design and build an automatic micromanipulator would have been unthinkable without the brilliant achievements in electronics that have been made during the past decade.

As one begins to analyze what is specifically demanded of a micromanipulator, the problem easily boils down to simple principles. All that one really needs in a micromanipulator is an arrangement whereby the tip of a micro-

needle or micropipette is either out of the way or in the exact position needed to perform the operation skillfully and with the least possible injury to the cell. Furthermore, as based on years of experience, practically any micrurgical problem can be solved with three microneedles or micropipettes.

To play it safe, we have designed an instrument capable of positioning four microneedles or micropipettes by servomechanism controls. Probably for the first time, the microscope and mechanical stage have been designed as an integral part of the micromanipulator. The focusing of the microscope, as well as adjustments of the stage, are also controlled by servomechanisms. The prototype of this new instrument, which is now being constructed, will be semiautomatic—in the sense that all movements can be activated by push-button controls. Later these controls will be replaced by punched-tape programming devices—by which photoelectric events in the cell or an amoeba, will automatically trigger the proper sequence of events to ensure the removal of a nucleus, a chromosome, or cytoplasm; having done this, a host cell will be brought into the field and the subcellular structure will be injected into it. This may appear to be wishful thinking, yet actually the electronic circuitry for such automatic control of microneedles is rather elementary, especially if one compares the difficulties encountered in moving-target indicators or the automatic tracking of missiles or satellites. Many of these problems have been elegantly solved.

Assuming that more intelligent experiments can be performed or—as Mazia so aptly states it—that more intelligent questions can be asked, the next important problem is to establish better lines of communication between the cell and the investigator. In any event, the cell must be made visible to the investigator, and this requires the application of microscopic optics. One important adjunct to the microscope and to the micromanipulator is closed-circuit television.⁸

The application of television to the microscope does not yield a better or prettier image on the video screen than can be obtained by conventional photomicrography, but much more can be done with the video image. This point will be discussed later.

Originally, the television adaptation was made with the intention of using ultraviolet light. Unfortunately the amount of ultraviolet light needed to form an adequate video picture is so great that the cells are damaged almost instantly and many are killed after a few minutes' exposure. The solution to the problem of instantaneous ultraviolet translation of images came from the development of the ultraviolet flying-spot microscope by Montgomery *et al.*⁹ By this method, cells remain alive and, in tissue culture, will go through their normal mitotic cycles even though they are illuminated by the potentially lethal ultraviolet light. With the development of better ultraviolet scanning tubes, the flying-spot microscope will produce excellent images of fine resolution. Moreover, the scanning program will be varied, so that the exposure of cells to ultraviolet light will become reduced to minimal levels.

Furthermore, through modifications in the scanning program imposed on the scanning tube, it is possible to illuminate intensely and hence irradiate selected

portions of any cell by lethal or physiologically effective radiation dosages. The introduction of this feature into the ultraviolet flying-spot microscope was described by Montgomery and Bonner.¹⁰

Video techniques applied to the microscope are especially valuable when combined with line selection and analysis procedures.¹¹ By these means, the image from the microscope is presented on a video screen as a series of horizontal scanning lines. The relative width of each line depends on the magnification employed. Even with moderate optical magnification, a single scanning line may represent less than one micron of the cell's structure. With appropriate delayed triggers, any one of the scanning lines can be isolated and displayed on a fast-rise cathode-ray oscilloscope. The selected portion of the image, a single line, then appears on the screen of the oscilloscope as pulses of varying widths, shapes, and amplitudes. These pulses represent merely the varying degrees of white or black equivalence picked up by the video tube from the microscope. An entire subcellular structure can be dissected electronically; from this a family of various pulse shapes is obtained.

Thus, better lines of quantitative communication can be established between the cell and the investigator. Following this, however, the problems of handling the results of such communications arise. If the video image can be dissected and presented as individual oscilloscope traces, is there any possibility that these data may be handled more rapidly? Can such data be stored and classified and, subsequently, recalled when needed? Again, based on present knowledge and experience, there is no question that facilities are already available for better and more efficient handling of such data.

The approach is the application of analogue and digital computer techniques equipped with memory storage, as has already been accomplished in the development of the Cytoanalyzer for scanning and interpreting exfoliated cancer cells.¹²

Returning to the conference on the biology of the amoeba to be held in 2158: What are some of the topics that should be incorporated in such a conference? In what ways will progress be made during the next 200 years to justify the inclusion of such topics in a conference? Progress is anticipated in five areas of research dealing with amoebae and related organisms.

(1) The Development of Chemically Defined Media for Culturing Amoeba

Promising developments in tissue culture media definitely point to the possibility that amoebae can also be propagated in simpler media, especially those that are organism-free. Certainly, a few years ago it seemed unlikely that vertebrate cells could ever be induced to grow in relatively simple media, especially when protein-free. The amoebae may always require proteins and other macromolecular organic food stuffs, but in these organisms, through the mechanisms of pinocytosis, the large molecules are able to enter the amoeba by processes independent of diffusion through the protoplasmic surface layer.

Unquestionably there are many desirable features inherent in amoebae that could be cultured without feeding them other organisms. As matters stand, well-fed amoebae have much "junk" inside them. To eliminate this, the

amoebae must be starved; even then, there is no assurance that the subcellular particles can always be correctly identified and classified as to those that belong to the amoeba or those that originally were parts of ingested organisms.

In all probability, all experimental work will be done on clones. Furthermore, there is no reason why strains of amoebae cannot be recognized and maintained on the basis of genetic markers. One need only to recall the work of Puck and his associates^{13a, b} on the somatic genetics of mammalian cells, cultured *in vitro*.

(2) *The Production of New Varieties of Amoebae Through Subcellular Transplantation*

This will require the development of better methods of transplanting nuclei although, with present techniques, there is adequate promise that much more spectacular results are definitely possible. Considerable attention should be given to the possible modification or creation of new varieties through hetero-specific and even heterogenetic transplantation of nuclei and other subcellular components. Again, clones and genetic markers will play an important role.

(3) *The Control of Growth and Division in the Amoebae*

The processes of growth and division should be amenable to independent control through various procedures, including, transplantation of subcellular structures, modification by nucleic acids or viruses introduced by microinjection, or the modifications induced by the action of nucleic acid depolymerases, both RNA and DNA. It should be possible to produce large amoebae by inducing growth and suppressing division. It should also be possible to stimulate nuclear divisions without growth and cell division and thus induce multinucleated organisms.

(4) *The Problems of Subcellular Ecology*

One aspect of this area is to establish a closer liason between biochemical activity and subcellular morphology. Moreover, the cytochemical aspects might even be brought down to submicroscopic levels. Some work is already being done on cytochemistry at submicroscopic levels using the electron microscope.^{14a-d} Perhaps, through these various procedures and others yet to be devised, the coupling of chemical energetics with the morphologic constituents primarily concerned with gelation may be worked out so precisely that such problems as amoeboid movement and cytokinesis can be clearly solved.

(5) *Natural and Induced Symbiosis*

The field of cellular biology is riddled with instances of unexpected symbiosis—largely because the symbionts may be submicroscopic, such as the kappa particles in the *Paramecium*. Undoubtedly other symbiotic relations may exist between the amoeba and microscopic as well as submicroscopic entities such as viruses. It is important in such studies to determine the precise role of the symbiont in the biology of the host cell. Some of the symbiotic forms, for example kappa particles, can be eliminated through the appli-

cation of agents such as nitrogen mustards, 2,6-diaminopurine, streptomycin, or chloromycetin.¹⁵

The alternative and equally important procedure to the creation of symbiont-free amoebae would be the creation of new symbiotic associations. One such approach has already been considered and some preliminary work has been done. The point here was to study the possibility of "infecting" the amoeba with an organism that possesses some of the characteristics of endogenous subcellular particles, notably, self-replication. One such organism that fulfills some of these requirements is the *Zoochlorella* that normally exists in symbiotic relationship with *Paramecium bursaria* and other organisms.

The symbiotic zoochloellae have the following useful features: they can be readily isolated and washed; they are large enough for microscopic study; they are small enough to be transplantable via micropipette transfer; natural "infection" in amoeba by them is improbable; an amoeba can be "infected" by direct implantation of zoochloellae into its cytoplasm; zoochloellae readily survive temporary sojourn outside their hosts; and they possess the main features of self-perpetuating cell inclusions.

The transplantation of zoochloellae from *Paramecium bursaria* to *Amoeba proteus* by microinjection techniques creates a direct contact between the particles and amoeba protoplasm. By ingestion, such particles are outside the cytoplasm and within a food vacuole, even though they are apparently inside the amoeba. In this connection, the pinocytosis reaction is similar to ingestion and, topologically, the material taken into the amoeba by such a mechanism is different from similar material introduced by careful microinjection. Such topologic differences need not necessarily lead to different end results, but they might do so.

The distinction between "infection" by ingestion or by subcellular transplantation can be further indicated by the methods of symbolic notation:^{5, 16}

ISOLATED AND WASHED ZOOCHLORELLAE

Ingested by amoeba

$$Qz_1 e V_2 g C_2 - N_2$$

Injected into amoeba

$$Qz_1 e C_2 - (N_2 v V_2)$$

TRANSPLANTED ZOOCHLORELLAE

From *Paramecium* to amoeba

$$Qz_1 v QC_1 e C_2 - (N_2 v V_2)$$

In the above symbolic statements, the following is the meaning of the symbols: *z*, zoochloellae; *C*, cytoplasm; *N*, nucleus; *V*, vacuole; *e*, element of; *g* subgroup of; $-$, not or excluding; *v*, join. Also, *Qz* means one or more zoochloellae and *QC* is a portion of cytoplasm. The subscript 1 refers to the *Paramecium*, while subscript 2 designates the amoeba.

Although amoebae can ingest paramecia containing symbiotic zoochloellae, "infection" of the amoeba by the symbiont rarely, if ever, occurs, since the

ingested plant cells are segregated within the food vacuole. Thus, following ingestion, the zoochlorellae are really outside of the amoeba, although apparently inside. Such zoochlorellae are subjected to death and digestion when inside the food vacuole. It is unlikely that a *zoochlorella* might escape through the vacuolar wall into the cytoplasm where it would become less susceptible to digestion and thus have a chance to remain alive and possibly reproduce.

On the other hand, if washed zoochlorellae are carefully implanted into the cytoplasm of an amoeba by microinjection, such symbionts are now in direct contact with the cytoplasm and now can be considered as xenic "subcellular inclusions." These inclusions are therefore no longer susceptible to the action of digestive mechanisms. In fact, these symbionts now have a chance of surviving and even reproducing.

Zoochlorellae, either individually or in known numbers, can be removed from immobilized *Paramecium bursaria* and placed into the cytoplasm of an amoeba by microinjection techniques. Alternatively, zoochlorellae can be removed from the *Paramecium* and washed in balanced salt solutions before implantation into the amoeba. Following implantation into the amoeba, the new incipient symbiotic system can be maintained in culture. Preliminary experiments have indicated that zoochlorellae will survive washing in Kassel's medium^{17a, b} and that this mixture of salts is readily tolerated by the amoeba cytoplasm following injection. The results of these preliminary experiments will be published later.¹⁸

The five areas mentioned above would seem to form the most logical basis for a conference on the amoeba in 2158. Unquestionably, there should be more advanced information 200 years from now; on the other hand, it is entirely likely that whoever may plan such a future conference will still have new horizons remaining for future explorations.

The amoeba has and will continue to play an important role in our subcellular and cellular studies. Perhaps one of the most regrettable features of the amoeba is its nuclear structures and the lack of chromosomal complexes such as are generally found in vertebrate and plant cells. It is unlikely that this defect may be remedied by then!

Furthermore, the nucleolar picture is obscure and so is the relationship between nucleoli and chromosomes. For example, we have no indication that there are such entities as nucleolar organizers and nucleolar chromosomes in the amoeba. If there are such entities, then we can claim that this line of communication between the amoeba and investigator has never been adequately established.

According to present information it does not seem likely that it will ever be possible to transplant subcellular fractions into the nucleus of the amoeba. There is a progressive deterioration of the nucleus of the amoeba, following mechanical puncture of the nuclear membrane and, in most instances, this leads to destruction of the nucleus and its expulsion from the amoeba. Insofar as we know, the amoeba does not have a phase in which the nuclear membrane disappears, as is true of many plant and animal cells during mitosis. In animal cells, methods have been worked out recently for implanting nucleoli

and even chromosomes prior to the reconstitution of the nuclear membrane during mitosis.¹⁹ These artificially introduced structures frequently become incorporated in the newly organized nucleus. By this method, the usual drastic injury to nuclei, following mechanical puncture of the membrane is by-passed.

The amoeba, therefore, has its limitations. However, much has been learned already about the biology of the amoeba and much of what has been learned can be applied profitably to other cells.

One point is certain: an important blending of micrurgical instrumentation, video or flying-spot circuitry, line selection and analysis, and the application of analogue computers is now feasible. Even now, information obtained from selected video scanning lines can be fed into computers for rapid analysis and classification of pulse heights and shapes; hence, nuclear densities and sizes, for example, are rapidly handled. These bits of information can become new lines of communication between the cell and investigator. The automatic micromanipulator is practically here.

Electronics has much to offer to biological research at cellular levels. Greater imagination in seeing the possibilities is needed, as well as someone who can design and build the instrumentation.

All these approaches cannot help but increase the amount of data obtainable from a single cell by one investigator. With such aids the researcher can then revert to and concentrate on the biological problems, confident that he will be able to get the maximum yield of reliable information from a single cell or single experiment. More important, the investigator can be relieved of much of the mechanical drudgery and thereby gain the time and energy to think of more intelligent questions to ask of the cells.

Perhaps then, greater strides can be made in solving the many riddles offered by living cells in general, and by amoebae in particular.

References

1. SCHAEFFER, A. A. 1926. Papers from Dept. Marine Biol. Carnegie Inst. Wash. **24**:(345).
2. BARBER, M. A. 1911. J. Infectious Diseases. **9**: 117.
3. MAZIA, D. 1956. Am. Scientist. **44**: 1.
4. KOPAC, M. J. & J. HARRIS. 1951. Anat. Record. **111**: 116.
5. KOPAC, M. J. 1957. Ann. N. Y. Acad. Sci. **68**(2): 380.
6. KOPAC, M. J. 1955. Trans. N. Y. Acad. Sci. **17**(3): 257.
7. KOPAC, M. J. 1929. Trans. Am. Microscop. Soc. **47**: 438.
8. KOPAC, M. J. 1955. Intern. Rev. Cytol. **4**: 1.
9. MONTGOMERY, P. O'B., W. A. BONNER & F. F. ROBERTS. 1956. Proc. Soc. Exptl. Biol. Med. **93**: 409.
10. MONTGOMERY, P. O'B. & W. A. BONNER. 1958. Scientific Am. **198**(5): 38.
11. KOPAC, M. J. 1959. In The Cell. Chap. 6. **1**: 161-190. J. Brachet and A. E. Mirsky, Eds. Academic Press. New York, N. Y. In press.
12. TOLLES, W. E. & R. C. BOSTROM. 1956. Ann. N. Y. Acad. Sci. **63**(6): 1211.
- 13a. PUCK, T. T. & H. W. FISHER. 1956. J. Exptl. Med. **104**: 427.
- 13b. TJIO, J. H. & T. T. PUCK. 1958. J. Exptl. Med. **108**: 259.
- 14a. RICHTER, G. W. 1958. J. Biophys. Biochem. Cytol. **4**: 55.
- 14b. FINCK, H. 1958. J. Biophys. Biochem. Cytol. **4**: 291.
- 14c. WATSON, M. L. 1958. J. Biophys. Biochem. Cytol. **4**: 475.
- 14d. PASTEELS, J. J., P. CASTIAUX & G. VANDERMEERSSCHE. 1958. J. Biophys. Biochem. Cytol. **4**: 575.

- 15a. STOCK, C. C., M. WILLIAMSON & W. E. JACOBSON. 1956. Ann. N. Y. Acad. Sci. **56**(5): 1081.
- 15b. BROWN, C. H. 1950. Nature. **166**: 527.
- 15c. GECKLER, R. P. 1949. Science. **110**: 89.
16. KOPAC, M. J. 1956. Ann. N. Y. Acad. Sci. **63** (6): 1219.
- 17a. KASSEL, R. & M. J. KOPAC. 1953. J. Exptl. Zool. **124**: 279.
- 17b. KASSEL, R. AND M. J. KOPAC. 1954. J. Exptl. Zool. **126**: 497.
18. KOPAC, M. J. Unpublished data.
19. KOPAC, M. J. & G. M. MATEYKO. 1958. Ann. N. Y. Acad. Sci. **73**(1): 237.

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